INVESTIGATOR’S BROCHURE

$^{64}$Cu-ATSM

Product: $^{64}$Cu-diacetyl-bis(N$^4$-methylthiosemicarbazone) (64Cu-ATSM)

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2. SUMMARY

2.1. Background
Since the 1930s, hypoxia (oxygen concentration of ≤ 1000 ppm) has been recognized as an important determinant of tumor biology in solid tumors. Hypoxia has been found in a wide range of tumors, including cervical cancer (1). Several studies have demonstrated that the pretreatment oxygenation status of the tumor can predict overall survival, disease-free survival, and/or local tumor control in patients with cervical cancer (1).

Several single-center studies have shown that the novel tracer, $^{60}$Cu-labeled diacetyl-bis($N^4$-methylthiosemicarbazone ($^{60}$Cu-ATSM), accumulates avidly in hypoxic tissues (2). Clinical studies using positron emission tomography (PET) with $^{60}$Cu-ATSM have demonstrated an inverse relationship between tumor uptake of this tracer and response to therapy in patients with lung carcinomas, and outcome in patients with cervical and rectal carcinomas (3-6).

2.2. Nonclinical Studies

The evolution of ATSM labeled with copper radionuclides (Cu-ATSM), and the in vitro, in vivo and clinical studies with this tracer, have been reviewed recently (2). The pre-clinical studies of Cu-ATSM have shown the agent to be selective for hypoxic cancers and ischemic myocardial tissue. Studies examining the hypoxia selectivity of Cu-ATSM have presented a number of mechanisms that explain the activity of this agent. From these studies and the clinical data (see below), it has been shown that Cu-ATSM is an effective agent for clinically delineating many hypoxic human malignancies by PET, but, as with all radiopharmaceuticals, it is not a universal agent. Animal studies have shown that care should be taken, in particular, with regard to the imaging of prostate tumors with Cu-ATSM.

Nonclinical pharmacology and toxicology safety studies have been performed: a single dose of the final formulation of 0.015 mg/person (0.008 mg/m², based on a 70-kg adult subject) was proposed for the clinical trials. In a cardiovascular and respiratory safety pharmacology study, dogs receiving a single dose of 0.300 mg/kg (6.00 mg/m²), a 750-fold higher dose than the proposed clinical dose, exhibited no treatment-related effects. In 14-day rat and rabbit toxicity studies, targeted maximum doses of 0.150 mg/kg/d (0.900 mg/m²/d) for rats and 0.060 mg/kg/d (0.720 mg/m²/d) for rabbits, did not produce any drug-related effects. These doses were approximately 110-fold (rats) and 90-fold (rabbits) higher than the maximum intended dose when compared on a once-daily dose basis and 1500-fold and 1200-fold higher when compared on a total-dose administered basis. These data demonstrated that the formulation has an appropriate margin of safety for clinical use. However, one or more of the components in the formulation preparation produced a modest (two-fold) increase of bacterial revertants, relative to the vehicle control, for one tester strain (TA100). It is important to note, however, that the concentration that produced this modest positive response is 70,000-fold higher than the maximum possible concentration that can be achieved in human plasma. The vehicle may have produced acute increases in blood pressures and heart rates of dogs, and minimal to mild tissue effects on the lung and skin (at the injection site) of rabbits.

2.3. Clinical Studies

To date, investigators at Washington University have evaluated 124 cancer patients (including non-small cell lung cancer [NSCLC], head and neck cancer, cervical cancer, breast cancer, brain tumor; and rectal cancer) with $^{60}$Cu-ATSM and found that the tumor uptake of $^{60}$Cu-ATSM was variable (See Section 7.0 for details) (3-7). In cervical cancer, these investigators have studied patients with locally-advanced cancer by $^{60}$Cu-ATSM-PET prior to initiation of therapy. $^{60}$Cu-ATSM-PET images were evaluated qualitatively and quantitatively by tumor/muscle ratio.
(T/M). The tumor in all patients but one showed measurable $^{60}$Cu-ATSM uptake on PET (with a mean ± standard deviation for T/M of 3.8 ± 2.0 (3)). Using log-rank analysis of the data, it was found that a T/M threshold of 3.5 was a statistically significant cut-off value that accurately distinguished patients whose cancer did not recur from those who developed a recurrence after completing therapy. Progression-free and cause-specific survival were significantly better in patients with T/M for $^{60}$Cu-ATSM ≤ 3.5 ($p = 0.006$ and $p = 0.04$, respectively) (3, 4). To document that $^{64}$Cu-ATSM images are of adequate quality and that the semiquantitative measure of tumor uptake is similar between $^{60}$Cu-ATSM and $^{64}$Cu-ATSM, the Washington University investigators compared the two agents in 10 patients with locally advanced cervical cancer. They found a good correlation between the uptake of $^{66}$Cu-ATSM and $^{64}$Cu-ATSM ($r = 0.95, P < 0.0001$) and comparable image quality; although, generally, the images with $^{64}$Cu-ATSM had a slightly better target-to-background ratio and tumors were delineated more clearly by comparison with the $^{66}$Cu-ATSM images. In addition, the pattern of uptake was found to be similar on the images obtained with the tracers during two different imaging sessions, indicating that the macroscopic distribution of hypoxia did not change greatly over this interval (1-9 days apart) (8). These studies support the feasibility of $^{64}$Cu-ATSM imaging and show the results are quantitatively similar to those obtained with $^{60}$Cu-ATSM, for which there is a larger body of experience.
3. LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS

ANP  authorized nuclear pharmacist
ALARA as low as reasonably achievable
CT  computed tomography
Cu-ATSM copper(II)-diacetyl-bis(N4-methylthiosemicarbazone)
DMSO  dimethyl sulfoxide
FMISO  $^{18}$F-fluoromisonidazole
FHV  fractional hypoxic volume
FOB  functional observational battery
ECG  electrocardiogram
mCi  millicurie
PET  positron emission tomography
PI  principal investigator
ROI  region of interest
SUV  standardized uptake value
SUV$_{\text{max}}$ maximum standardized uptake value
T/M  tumor/muscle activity ratio
VOI  volume of interest
4. INTRODUCTION

4.1. Tumor Hypoxia

Since the 1930s, hypoxia (oxygen concentration of ≤ 1000 ppm) has been recognized as an important determinant of tumor biology in solid tumors. Hypoxia has been found in a wide range of tumors, including cervical cancer (1). Several studies have demonstrated that the pretreatment oxygenation status of tumors can predict overall survival, disease-free survival, and/or local tumor control in patients with cervical cancer (1).

Numerous animal studies have confirmed that hypoxia contributes to radiation resistance (9-11). There is evidence suggesting that tumor hypoxia contributes to resistance to standard radiation therapy, some chemotherapy, and chemoradiation, and subsequently, to poorer clinical outcome (12). Studies of tumors transplanted into rodents have shown that the proportion of cells in the hypoxic fraction of such tumors increases with tumor size and may range from < 10% to up to 50% of the total viable cell population (13). The level of oxygen delivery can alter these tumors' response to radiation (13-16). Disrupting oxygen delivery increases radiation resistance, while improving it increases tumor radiosensitivity (17-28). Overview analysis of over 10,000 patients in 83 randomized trials conducted up to 1995 showed that improvement in tumor oxygenation significantly improves locoregional disease control with an odds ratio of 1.21 (95% confidence interval 1.12-1.30) and overall survival with an odds ratio of 1.13 (95% confidence interval 1.05-1.21) (29). Clinical studies continue to show that hypoxia in human tumors contributes to therapy failure (30-35).

Resistance to some chemotherapeutic agents also has been attributable to hypoxia (36-38). The exact mechanism is not known, but several mechanisms have been proposed. These include differential uptake and metabolism of drugs by hypoxic versus fully oxygenated cells (38, 39), the reduced proliferation of hypoxic tumor cells (40, 41) and slower progression of hypoxic cells through the cell cycle (42-44). The effectiveness of chemotherapy can be improved either by enhancing oxygen delivery to a tumor or by using hypoxia to selectively activate drugs (45-47).

There is direct and indirect evidence that human tumors contain hypoxic cells, and these cells are considered to affect tumor behavior and response to therapy. Measurement of hypoxia is possible with invasive methods including polarographic needle electrodes, exogenous markers of hypoxia (such as the 2-nitroimidazole, pimonidazole), and endogenous markers (such as carbonic anhydrase-IX), as well as noninvasive imaging techniques. These methods, except for imaging, are invasive and require direct access to tumor or analysis of tumor biopsy material. Some markers, for example pimonidazole, require injection of the drug prior to biopsy. The invasive nature of these methods makes it difficult to assess hypoxia routinely in a clinical setting.

4.2. Imaging of Hypoxia

Investigations over the past 15 years have led to quantitative, non-invasive methods for radionuclide imaging of hypoxia, particularly by positron emission tomography (PET) (48, 49). Much of the work in hypoxia imaging by PET has used labeled nitroimidazoles, a class of compounds whose metabolism and tissue retention is dependent upon the state of tissue oxygenation. After entering a viable cell, nitroimidazoles are reduced to RNO₂ radicals,
regardless of intracellular oxygen concentration. In the presence of tissue oxygen, the radical is immediately re-oxidized to superoxide, and the original uncharged compound leaves the cell. If intracellular oxygen levels are low, however, the RNO₂ radical is further reduced to a more reactive form, which binds covalently to intracellular macromolecules and remains within the cell. The nitroreduction that occurs in hypoxic cells is believed to be enzyme mediated (50-52), and nitroimidazole compounds have a high affinity for xanthine oxidase, a nitroreductase (53). The most extensively studied radiolabeled nitroimidazole for in vivo imaging is ¹⁸F-fluoromisonidazole (FMISO), which is lipophilic and therefore diffuses readily through cell membranes. Cell culture studies and in vivo studies using a variety of transplanted rodent animal tumors have shown that intracellular retention of FMISO is dependent on oxygen concentration, and that the rate of FMISO binding can be up to 28 times higher under hypoxic conditions than under normoxic conditions (50, 52, 54-56). Based on in vivo FMISO biodistribution studies in animals and humans, tissue hypoxia has been defined as an FMISO tissue-to-blood ratio ≥ 1.2 by 2 hrs after radiotracer administration (56). Elevated ratios (≥ 1.4) have been observed in tumors, and were used to estimate the “fractional hypoxic volume” (FHV) (56). Rasey et al. used FMISO to study 37 cancer patients before therapy (57). They observed hypoxia in tumors of 36 of the 37, and FHVs ranged from 0% to 94.7%. The distribution of hypoxia was heterogeneous and the extent of hypoxia varied markedly between tumors in the same site or of the same histology. In a recent study, Rajendran et al. have shown that the results of pre-therapy FMISO-PET were predictive of survival in patients with head and neck cancers (58). To date, there is no established method for identifying patients who will benefit from hypoxic-directed therapy. However, Rischin et al. recently demonstrated that FMISO-PET is useful in directing hypoxia-specific treatment in patients with head and neck cancer; the uptake of FMISO predicted the greater effectiveness of tirapazamine therapy (59). In this study, only patients with increased FMISO uptake benefited from the addition of tirapazamine to radiotherapy. This is very important considering the side effects of tirapazamine. Iodinated tracers, such as ¹²³I-iodoazomycin arabinoside, also have been used to assess hypoxia by single-photon imaging methods in preclinical and pilot clinical studies (60). Several other potentially hypoxia-measuring radiopharmaceuticals labeled with F-18 or Tc-99m have been developed, and are currently being evaluated in animal models and patients with solid tumors (61-65).

An alternative approach to hypoxia imaging has relied upon the use of radiometal chelates. This method offers the potential for preparation of the radiopharmaceutical by “kit” labeling of a stable precursor with a positron-emitting radionuclide such as ⁶⁰Cu or ⁶⁴Cu. This is analogous to the way most ⁹⁹mTc compounds are produced in clinical nuclear medicine practice, and offers the possibility of a hypoxia-imaging radiopharmaceutical with widespread availability. One such radiometal chelate is copper(II)-diacetyl-bis(N⁴-methylthiosemicarbazone) (Cu-ATSM) developed by Fujibayashi and colleagues (2-6, 66-69).

4.3. Clinical Imaging with Cu-ATSM

To date, investigators at Washington University have evaluated 124 cancer patients (Including NSCLC, head and neck cancer, cervical cancer, breast cancer, brain tumor, and rectal cancer) with ⁶⁰Cu-ATSM. The investigators found that the uptake of ⁶⁰Cu-ATSM was heterogeneous in these cancers. The kinetic studies demonstrated that the images can be easily evaluated quantitatively by T/M. In NSCLC, the uptake of ⁶⁰Cu-ATSM was predictive of response and in rectal cancer; the uptake of ⁶⁰Cu-ATSM was predictive of prognosis (See Section 7.0 for details) (3-6).
In cervical cancer, these investigators have studied patients with locally-advanced disease by $^{60}$Cu-ATSM-PET prior to initiation of therapy. $^{60}$Cu-ATSM-PET images were evaluated qualitatively and quantitatively by tumor/muscle ratio (T/M). The tumor in all patients but one showed measurable $^{60}$Cu-ATSM uptake on PET (with a mean ± standard deviation for T/M of 3.8 ± 2.0 (3).

To document that $^{64}$Cu-ATSM images are of adequate quality and that the semiquantitative measure of tumor uptake is similar between $^{60}$Cu-ATSM and $^{64}$Cu-ATSM, the Washington University investigators compared the two agents in 10 patients with locally advanced cervical cancer. A good correlation was observed between the uptake of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM ($r = 0.95, P < 0.0001$). The image quality was comparable, although the images with $^{64}$Cu-ATSM generally had a better target-to-background ratio; and tumors were seen more clearly in most cases than with $^{60}$Cu-ATSM. Importantly, the pattern of uptake was similar on the images obtained with the tracers during two different imaging sessions 1 to 9 days apart, indicating that the macroscopic distribution of hypoxia did not change greatly over this interval (8). These studies support the feasibility of $^{64}$Cu-ATSM imaging and show the results are quantitatively similar to those obtained with $^{60}$Cu-ATSM, for which there is a larger body of experience.
5. PHYSICAL, CHEMICAL, AND PHARMACEUTICAL PROPERTIES AND FORMULATION

5.1. Drug substance

5.1.1. Names

$^{64}\text{Cu-ATSM}$

5.1.2. Pharmaceutical class

Small molecule

5.1.3. Chemical Structure

![Chemical Structure](image)

5.2. Drug Product

5.2.1. Names

Cu-ATSM

5.2.2. Physical Characteristics

Colorless/pale yellow solution

5.2.3. Active Ingredient

$^{64}\text{Cu-ATSM}$ is a positron-emitting radiopharmaceutical for use in conjunction with PET imaging. The half-life of $^{64}\text{Cu-ATSM}$ is 12.7 hours.

5.2.4. Dose Form and Route of Administration

18-25 mCi of $^{64}\text{Cu-ATSM}$ will be administered intravenously as a bolus.

5.2.5. How Supplied

$^{64}\text{Cu-ATSM}$ will be made on-site as an isotonic, sterile, pyrogen-free, clear and colorless/pale yellow solution. $^{64}\text{CuCl}_2$ solution (up to 100 mCi) will be supplied in 10-200 μL of 0.1 M HCl by Washington University School of Medicine. The $^{64}\text{CuCl}_2$ will have been sterile filtered and tested for pyrogens prior to shipping. A sterility test will have been conducted and results will follow when completed. Radionuclidic impurities that are present will be documented and provided to the recipient by a fax prior to shipping. H$_2$ATSM kits will be supplied by Proportional Technologies, Inc (Houston, TX), and sent to recipient by Washington University School of Medicine along with the $^{64}\text{CuCl}_2$ solution.

The Proportional Technologies, Inc. H$_2$ATSM ligand kit is designed for Cu-64 labeling and is produced and stored as a lyophilized vial preparation. It is re-hydrated with 10 mL
of specially designed reconstitution solution at the clinical sites. The reconstituted ligand solution is stable and ready for labeling with commercially available Cu-64. The labeling process is simple and rapid, requiring less than one minute of mixing the reconstituted ligand with Cu-64 solution at room temperature. The labeled $^{64}$Cu-ATSM solution is stable for more than 8 hours at room temperature and ready for patient injection.

This H$_2$ATSM ligand kit includes two components, H$_2$ATSM lyophilized ligand vial (shown in Figure 1) and a sealed vial containing 10 mL of reconstitution solution. Each ligand vial contains 15 µg of H$_2$ATSM and 200 mg of sucrose as excipient. The empirical formula of H$_2$ATSM is C$_{8}$H$_{16}$N$_{6}$S$_{2}$ and the calculated molecular weight is 260.4. The reconstitution solution for the kit is composed of 10% propylene glycol, 25 mM sodium acetate solution. Propylene glycol is required to increase the solubility and stability of H$_2$ATSM ligand in the reconstitution solution. Sodium acetate is employed to maintain pH for the labeling reaction, as Cu-64 is delivered in diluted HCl solution (pH~1). A volume of 10 mL of reconstitution solution provides a >2 fold excess of buffer for up to 1.2 ml of pH 1 HCl.

Figure 1:
Lyophilized H$_2$ATSM kit for labeling with Cu-64

The quantitative composition of the drug product prior to Cu-64 labeling is shown below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetyl bis(N$^4$-methylthiosemicarbazone) (H$_2$ATSM)</td>
<td>15x10$^{-3}$ mg</td>
</tr>
<tr>
<td>Sodium Acetate (NaOAc)</td>
<td>20 mg</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200 mg</td>
</tr>
<tr>
<td>propylene glycol</td>
<td>1.05 g</td>
</tr>
<tr>
<td>SWFI</td>
<td>~9 g</td>
</tr>
</tbody>
</table>

The specific activity of the $^{64}$Cu is routinely determined by an isotope dilution technique previously described (McCarthy DW, Shefer RE, Klinkowstein RE, et al. Efficient production of high-specific-activity $^{64}$Cu using a biomedical cyclotron. Nucl Med Biol 1997; 24:35-43.). Significantly higher specific activities of Cu-64 are now being obtained than in the past, with recent values (n=5) calculated to the end of bombardment averaging 1,588 mCi/µg (± 307 mCi/µg [SD]), and ranging from 1247-2008 mCi/µg. Assuming, that the time of administration of the $^{64}$Cu-ATSM will range from 4 to 36 hours after the end of bombardment, the mass of $^{64}$Cu-ATSM will range from 0.06 to 0.7 µg per 25 mCi dosage.

5.2.6. Storage and Handling
$^{64}$CuCl$_2$ solution should be stored upright in an appropriate lead or tungsten alloy-shielded container at room temperature, if required. Handling should be done using techniques to keep personnel radiation exposure ALARA, by minimizing handling time.
and placing adequate shielding between personnel and activity, as well as using tools to increase distance from activity. The $^{64}$Cu-ATSM must be administered within three hours of preparation.

5.2.7. Preparation and Administration

$^{64}$Cu-ATSM will be produced on-site by the preferred method of adding the contents of the GMP-produced, pyrogen-free H$_2$ATSM kits to the shielded vial containing $^{64}$CuCl$_2$ solution. This is the PTI Kit Formulation (Version 2) described in Appendix 1. A previously described alternative method (PTI Kit Formulation (Version 1)) adds the radioactive$^{64}$CuCl$_2$ solution to the H$_2$ATSM kit (Appendix 2). The kit contains H$_2$ATSM and an injection-grade sugar. This preparation will be performed with sterile, pyrogen-free reconstitution solutions supplied in a separate glass vial with septum access by the manufacturer of the kit. Following reconstitution, the final $^{64}$Cu-ATSM will be in an isotonic solution with pH ~6.4. The radiochemical purity will be determined preferably with use of radiochromatography (Appendix 3), but can also be measured with the alternative methodology involving Oasis cartridge analysis (Appendix 4). The final $^{64}$Cu-ATSM will be provided as an isotonic, sterile, pyrogen-free, clear and colorless solution.

6. NONCLINICAL STUDIES

6.1. Preclinical Studies

The evolution of Cu-ATSM, and the in vitro, in vivo and clinical studies with this tracer, have been reviewed recently (2). In 1999, the in vitro kinetics of $^{64}$Cu-ATSM in EMT6 cells were compared to those of FMISO, the misonidazole drug described earlier (67). Uptake of $^{64}$Cu-ATSM, $^{64}$Cu-PTSM and FMISO into EMT6 cells was investigated at dissolved oxygen concentrations of 0 (anoxia), 1×10$^3$, 5×10$^3$, 5×10$^4$ and 2×10$^5$ (normoxia) ppm. This study showed a sigmoidal inflection (i.e., the threshold of selectivity) between 5×10$^3$ (3.8 mm Hg) and 1×10$^3$ ppm (0.8 mm Hg) of dissolved O$_2$ which is centered around pO$_2$ levels of tumor hypoxia (2-3 mm Hg). FMISO also showed oxygen concentration-dependent uptake, but with lower percentages than $^{64}$Cu-ATSM, and $^{64}$Cu-PTSM showed 83-85% uptake into the cells after 1 hr, independent of O$_2$ concentration. Compared to FMISO, Cu-ATSM exhibited more efficient uptake and faster washout kinetics in hypoxic and normoxic cells offering the possibility of a superior means of detecting tumor hypoxia by PET imaging.

In 1999, a comparative biodistribution study of $^{64}$Cu-ATSM with $^{64}$Cu-PTSM in BALB/c mice bearing EMT6 tumors was reported (67). The biodistribution data of $^{64}$Cu-ATSM and $^{64}$Cu-PTSM showed optimal tumor uptake after 10 min post injection, suggesting a rapid trapping mechanism for Cu-ATSM in solid tumors. Ex vivo autoradiography of tumor slices following co-injection of $^{60}$Cu-PTSM and $^{64}$Cu-ATSM into the same animal showed uniform $^{60}$Cu-PTSM uptake throughout the EMT6 tumor but heterogeneous uptake of $^{64}$Cu-ATSM, consistent with trapping of $^{64}$Cu-ATSM in the ‘hypoxic’ regions of the tumors. Using oxygen needle electrode measurements of the solid tumor, PET and electronic autoradiography, a strong relationship between low tumor pO$_2$ and high Cu-ATSM accumulation was observed in 9L gliosarcoma tumors in rats (68). By chemical manipulation of tumor pO$_2$, a significant increase in Cu-ATSM was observed in hypoxic-induced tumors. This was the first study confirming that Cu-ATSM uptake in cancerous tissues in vivo was dependent upon the tissue pO$_2$. 
One of the most important validation studies of Cu-ATSM as an agent for delineating hypoxia was reported in 2006 by Yuan et al. (69). The authors compared the autoradiographic distributions of $^{64}$Cu-ATSM with that of a well-established hypoxia marker drug in R3230 mammary adenocarcinomas, fibrosarcomas (FSA), and 9L gliomas. There was close correlation of $^{64}$Cu-ATSM uptake and hypoxia in R3230Ac and 9L tumors, but not in FSA tumors. The same relationship was observed with 2 other hypoxia markers, pimonidazole and carbonic anhydrase IX, in the FSA tumors. This study strongly confirmed that $^{64}$Cu-ATSM on a histological level is a valid PET hypoxia marker in most tumor types.

The retention mechanism of Cu-ATSM has been hypothesized and explored by a number of groups (70-80). Two major mechanisms have been proposed. A proposed mechanism of Cu-ATSM retention was first reported by Fujibayashi et al., where it was suggested that Cu(II)-ATSM reduction only occurred in hypoxic cells and that the resultant Cu(I) was irreversibly trapped (66). Additional studies on the mechanism of Cu-ATSM retention were reported by Dearling et al. (70) and Maurer et al. (78). These reports suggested that reduction of Cu(II)-ATSM took place in both normoxic and hypoxic cells resulting in unstable Cu(I)-ATSM. This unstable species would slowly dissociate, and if completely dissociated (in hypoxic cells), it would be irreversibly trapped, but in the presence of oxygen (normoxic cells), the Cu(I)-ATSM would be reoxidized to Cu(II)-ATSM and diffuse back out.

In summary, pre-clinical studies of Cu-ATSM, labeled with a positron-emitting radionuclide of copper ($^{60}$Cu, $^{61}$Cu, $^{62}$Cu or $^{64}$Cu), have shown the agent to be selective for hypoxic cancers. Mechanistic studies examining the hypoxia selectivity of Cu-ATSM have presented a number of mechanisms that explain the activity of this agent. From these studies and the clinical data (see below) it has been shown that Cu-ATSM is an effective agent for clinically delineating many hypoxic human malignancies by PET, but, as with all radiopharmaceuticals, it is not a universal agent. Animal studies have shown that care should be taken in particular regard to the imaging of prostate tumors with Cu-ATSM (2).

6.2. Nonclinical Toxicology and Pharmacology

Agreement with the Food and Drug Administration was obtained in Pre-IND 62,675 discussions regarding the formulation of agent to be tested. The batch formulation approved was a worst-case scenario, based on the final composition of $^{64}$Cu-ATSM at the time of injection into humans. The formulation contained the following components: 2.1556 g H$_2$ATSM (69.73%), 153.9 mg non-radioactive Cu-ATSM (4.98%), 12.0 mg CoCl$_2$ (0.38%) and 0.77 g NiCl$_2$ (24.91%). These four solid materials were ground and mixed to produce a homogenous mixture. The solid formulation was then dissolved in DMSO (1%), ethanol (5%) and saline (94%) prior to use in the preclinical toxicology studies.

6.2.1. Mutagenicity/Cytotoxic studies

In Vitro Salmonella Reverse Mutation Plate Incorporation Assay (68, 81, 82). The potential mutagenic activity of the formulation was investigated in the Salmonella Reverse Mutation Plate Incorporation Assay, which is an in vitro test designed to detect point mutations in bacterial tester strains induced by chemical agents. The mutagenic events are reverse mutations that cause histidine-requiring mutants to revert to their prototrophic (non-histidine requiring) state. The study was conducted in two parts such that an initial mutagenicity/cytotoxicity assay was conducted first, which was followed by a confirmatory assay. For both assays, the formulation was tested in S. typhimurium strains TA68, TA100, TA102, TA135, and TA1537 in the presence and absence of S9 metabolic activation. These strains are routinely used to evaluate preclinical toxicity.
(mutagenicity) of compounds with well-documented methods (68, 82). Concentrations of 0, 10, 25, 30, 40 and 100 µg/plate were used for the initial mutagenicity/cytotoxicity assay, whereas 0, 10, 40, 100, 500, 1000 µg/plate were used in the confirmatory assay.

In the range-finding assays, the formulation did not exhibit a dose-related mutagenic response in any of the five tester stains in the presence or absence of S9 metabolic activation, and there was no evidence of cytotoxicity for any strain in the presence or absence of S9 metabolic activation. With the lack of cytotoxicity and visible precipitate at concentrations up to 100 µg/plate, the concentration range was increased to include 500 and 100 µg/plate. A modest (two-fold) increase of revertants relative to the vehicle control was observed for strain TA100 in the absence of S9 at 1000 µg/plate.

**In vitro L5178Y/TK<sup>−/−</sup> Mouse Lymphoma Mutation Assay (68, 81, 83, 84).** This assay was conducted to evaluate the mutagenic potential of the formulation in a mammalian cell system. The L5178Y/TK<sup>−/−</sup> cell line is sensitive to the cytotoxic effects of the pyrimidine analog, trifluorothymidine (TFT), and when treated *in vitro* with mutagenic or carcinogenic agents, TK<sup>−/−</sup> is mutated to the TK<sup>+/−</sup> genotype which confers TFT-resistance. The mutant cells then proliferate and form colonies when cloned in selective medium containing TFT. This assay is used to detect gene alterations caused by compounds with well-documented methods. This assay was performed in three parts; the cytotoxicity experiment was conducted first to determine the concentration range for the mutagenicity experiments. The initial mutagenicity experiment was performed next, and to confirm negative or positive results from the initial mutagenicity experiment, a confirmatory (definitive) experiment was performed. The formulation exposures were for 4 hours in the presence and absence of an S9 activation system for the cytotoxicity and initial mutagenicity experiments. For the confirmatory experiment, exposures were for 4 hours in the presence of S9 and 24 hours in the absence of S9. Cultures from the initial and confirmatory mutagenicity experiments which demonstrated a relative suspension growth (RSG) of more that 10% were cloned in triplicate in restrictive medium to select for the mutant phenotype, following a two day expression period. After a 12-15 day selection period, mutant colonies for positive controls, solvent controls and positive test article responses (should any exist) were enumerated.

Limited solubility of the formulation stock solution in culture media and the requirement to keep the final concentration of vehicle to 1% or less restricted the maximum dose to 100 µg/mL in the cytotoxicity experiment. The results of this first experiment showed that concentrations ≥25 µg/mL in both the presence and absence of S9 were excessively toxic (>90% toxic relative to control). No cultures were cloned from this experiment. In the initial mutagenicity experiment, concentrations 0, 3 (without S9 only), 5, 7, 10, 12, 15, 18 20 and 25 µg/mL were tested in the presence and absence of S9. The results of this experiment indicated excessive cytotoxicity with the formulation concentrations ≥15 µg/mL in the absence of S9, and ≥20 µg/mL in presence of S9. The formulation was non-mutagenic in both the absence and presence of metabolic activation, as the mutation frequency was not elevated by a factor of two or more times the vehicle mutation frequency at any of the concentrations tested. In the confirmatory assay, the formulation concentrations of 0, 0.5, 1, 3, 5, 10, 12 and 15 µg/mL were tested without S9 (24 hr exposure), and 0, 10, 12, 15, 18 and 20 µg/mL were tested with S9 (4 hr exposure). In the absence of S9, excessive cytotoxicity was achieved with concentrations ≥10 µg/mL, whereas, the remaining concentrations exhibited a range of cytotoxic effects (41-88% toxic), relative to control. Excessive cytotoxicity was not observed in the presence of S9 in the confirmatory assay, instead the cytotoxicity ranged
from nontoxic to 72% toxic, relative to control. No statistically significant (p<0.05) or dose-related increase in mutation frequency was observed at any of the formulation concentrations tested, and none of the concentrations produced a mutant frequency that was 2-fold or higher than the solvent control. The positive controls (methyl methanesulfonate and Benzo[a]pyrene) exhibited statistically significant increases in mutation frequency, which were 2-fold or higher than the solvent control, showing that the assay was sensitive to detecting mutagenicity. Under the conditions of the study, the formulation was negative in the L5178Y/TK+/- Mouse Lymphoma Mutagenesis Assay.

In Vivo Micronucleus Assay in Rats. The potential for the formulation to induce chromosomal aberrations and spindle malformations in vivo was evaluated using the Micronucleus Assay in conjunction with the 14-day toxicity study of the formulation in rats (below). Fischer 344 rats (15/sex/group) were treated with intravenous doses of 0, 0.075 and 0.150 mg/kg/d (0, 0.40 and 0.900 mg/m²/d) once a day for 14 consecutive days. Bone marrow was collected on study days 15 (10 rats/sex/group) and 29 (5 rats/sex/group) for preparation of bone marrow smears; however, only the day 15 smears were evaluated. Cytogenetic damage is indicated by the presence of micronuclei in polychromatic erythrocytes.

No statistically significant (p<0.05) or dose-dependent increase in micronucleated polychromatic erythrocytes was noted for rats treated with the formulation relative to the vehicle control groups on day 15. Rats treated with cyclophosphamide (positive control; males only) exhibited a statistically significant increase in micronucleated polychromatic erythrocyte counts on day 15, as compared to the vehicle control group. This demonstrated that the assay was sensitive to detecting mutagenicity. Since there was no apparent formulation-related effect at the end of the treatment period, bone marrow slides that were prepared from rats (recovery animals) on day 29 were not evaluated. The formulation was concluded to be negative in the rat micronucleus test.

6.2.2. Acute Cardiovascular and Neurological Safety Study
Cardiovascular and Pulmonary Safety Testing in Beagle Dogs. The objective of this study was to evaluate the cardiovascular and pulmonary safety of the toxicology formulation when administered as a single intravenous bolus dose in Beagle dogs (2/sex/dose group). The formulation was dissolved initially in dimethyl sulfoxide (DMSO) and then diluted with ethanol and 0.9% sodium chloride such that the final concentration of the test article, DMSO, ethanol and sodium chloride, USP were 0.03 mg/mL, 0.3% (v/v), 7% (v/v), and 92.7% (v/v), respectively. A targeted dose of 0.300 mg/kg (6.00 mg/m²) was administered to treated dogs, while control animals received an equal volume (10 mL/kg) of vehicle. Cardiovascular data (systemic arterial blood pressure, heart rate, and electrocardiogram (ECG) waveforms) were collected for up to 60 hours post dosing via implantable radiotelemetry units. ECG interval measurements were made on the ECG waveforms. Pulmonary data (respiratory rate, tidal volume and minute volume) were collected continuously for approximately 4-5 hours post dosing. Clinical observations, body temperatures and clinical pathology were also evaluated.

No deaths occurred in this study and there were no formulation-related abnormal clinical observations. However, excessive salivation was observed for one control dog and one test-article treated dog which may have been due to the DMSO component of the formulation. During the study there were no changes in body weights or body temperature, and there were no changes in hematology or clinical chemistry, including C-reactive protein, serum amyloid A and troponin T. There were no alterations in heart
rate, blood pressure, or ECG that could be attributed to the administration of the toxicology formulation. However, vehicle-related acute blood pressure and heart rate alterations were observed during the first 10 minutes post dosing. Blood pressures were increased up to 15 mm Hg, and heart rates were increased up to 30 BPM, for both vehicle-dosed and drug-dosed animals. On average, blood pressures returned to near baseline values after 10 minutes, whereas, heart rates gradually returned to baseline values over the first 1.5 hours post-dose. These cardiovascular effects may have been due to the DMSO component in the formulations. Evaluation of ECG interval data revealed no apparent or statistically significant differences in the test group as compared to vehicle, and there were no alterations in ECG rhythm or morphology. Finally, there were no apparent or statistically significant alterations in respiratory rates, tidal volume or minute volume in this study.

**Neurological Safety Assessment in Rats.** Neurotoxicology assessments [functional observational battery (FOB) evaluations] for rats were done in conjunction with the 14-day toxicity study of the formulation in rats (below). Rats (15/sex/group) were treated intravenously with the formulation at doses of 0, 0.075 and 0.150 mg/kg (0, 0.450 and 0.900 mg/m²), once a day for 14 consecutive days. Neurological toxicity evaluations were conducted on rats prior to dosing (pre-study), and on days 14 (10/sex/group) and 28 (recovery rate; 5/sex/group). The following FOB parameters were monitored: home cage observation (e.g. tremors, convulsions, biting, vocalizations, posture, fur appearance) arousal/anxiety when handheld, open field mobility/gait, reactivity to sensory stimulation (e.g. visual, auditory, tactile, pain), hindlimb extension, catalepsy, forelimb and hindlimb grip strength, righting reflex, footsplay, body temperature and body weights.

No treatment-related, toxicologically significant or otherwise noteworthy changes were observed for any of the FOB parameters evaluated at pre-study (baseline) or on days 14 and 28.

**6.2.3. Subacute Toxicity Studies**

**14-Day Toxicity Study of Toxicology Formulation in Rats.** The objective of this study was to evaluate the target organ toxicity of the formulation and its reversibility when administered intravenously to rats. Male and female Fischer 344 rats (15/sex/group) were administered the formulation intravenously at target dose levels of 0.075, and 0.150 mg/kg (0.450 and 0.900 mg/m²) once a day for 14 consecutive days. Control rats received an equal volume (5 mL/kg) of vehicle (mixture of DMSO, ethanol and 0.9% sodium chloride for injection USP at final concentrations of 0.3% v/v, 7% v/v and 92.7% v/v, respectively) once a day for 14 days. Cage-side clinical observations were performed and recorded daily, and hand-held physical and clinical observations were performed pretest, and weekly throughout the treatment and recovery periods. Body weights were measured twice weekly during the treatment and recovery periods. Hematology and clinical chemistry parameters were evaluated on days 8, 15 and 29. FOB tests to determine neurological toxicity were performed pretest, and on days 14 and 28. Organ weights were determined on days 15 and 29. A bone marrow micronucleus assay was conducted using bone marrow from rats euthanized on day 15. Microscopic histopathology was performed on tissues from rats in the high does (0.150 mg/kg/d) and control groups that were euthanized on day 15.

The results are summarized in Table 1. Intravenous administration of the formulation at target dose levels of 0.075 or 0.150 mg/kg/d for 14 consecutive days did not result in any
clearly dose-related, treatment-related and/or toxicologically significant effects on mortality, clinical observations, mean body weights, mean body weight gains, hematology, clinical chemistry, neurotoxicity (as measured by FOB), mutagenicity (bone marrow micronucleated erythrocytes) or organ weights. No treatment-related gross or microscopic lesions were observed in any of the tissues evaluated. Based on these finding, the no-observed-adverse-effect level (NOAEL) for the study was 0.150 mg/kg/d (0.900 mg/m²/d). This dose is approximately 110-fold higher than the maximum intended human dose.

14-Day Toxicity Study of the Toxicology Formulation in Rabbits. The results are summarized in Table 1. The objective of this study was to determine target organ toxicity of the formulation and its reversibility when given intravenously to rabbits twice daily (BID) for 14 consecutive days. Male and female New Zealand White rabbits were administered the toxicology formulation intravenously twice a day for 14 days at target dose levels of 0.030 and 0.060 mg/kg/d (0.360 and 0.720 mg/m²/d). Control rabbits received an equal volume (1 mL/kg) of vehicle (mixture of DMSO, ethanol and 0.9% sodium chloride for injection, USP at final concentrations of 03.% v/v, 7% v/v and 92.7% v/v, respectively) twice a day for 14 days. Clinical signs of toxicity were monitored daily, and body weights were measured twice weekly during the treatment period and once weekly during the recovery period. Hematology and clinical chemistry parameters were evaluated on days 8, 15 and 29. Microscopic histopathology was performed on tissues from all rabbits in the high doses (0.06 mg/kg/d) and control groups that were euthanized on day 15.

The results are summarized in Table 1. Intravenous injection of the formulation at target dose levels of 0.030 and 0.060 mg/kg/d to male and female rabbits for 14 days resulted in no premature or unscheduled deaths. No adverse clinical signs were observed, and no treatment-related effects on body weight, body weight gain, hematology, clinical chemistry, or absolute and relative organ weights were seen. No drug-related gross lesions were observed in any male or female rabbit, and no drug-related histopathological effects were observed in any rabbit dosed at 0.060 mg/kg/d at the end of the 14 day dosing period. The no-observed-effect level of toxicology formulation was 0.060 mg/kg/d (0.720 mg/m²/d), which is 90-fold higher than the maximum intended human dose. However, it was noted that the vehicle may have produced minimal to mild effects on the lung and skin (at the injection site) tissues.
Table 1. Summary of 14-day toxicity studies with the Cu-ATSM/H₂ATSM formulation in rats and rabbits. Animals were given a daily dose of the formulation i.v. for 14 days. All the studies were conducted in compliance with GLP regulations (21 CRF, Part 58).

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of Animals/ Sex/Dose</th>
<th>Daily Dose</th>
<th>Total Dose</th>
<th>Target Organs/Systems of Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fischer 344 Rats</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg/kg/day</td>
<td>mg/m²/day</td>
<td>mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.075</td>
<td>0.450</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.150</td>
<td>0.900</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand White Rabbits</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
<td>0.36</td>
<td>0.42</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>0.06</td>
<td>0.72</td>
<td>0.84</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

6.2.4. Biodistribution and Radiation Dosimetry

Human estimates for ⁶⁰Cu-ATSM and ⁶⁴Cu-ATSM were calculated from ⁶¹Cu-ATSM rat biodistribution data. The biodistributions were determined in mature female Sprague-Dawley rats (weights 141-180g, Charles River Laboratories, Inc., Wilmington, MA, USA) at 5, 15 and 30 minutes and at 1, 2, and 4 hours post-injection (n=4 per time points). Nineteen organs were harvested, weighed and then counted for radioactivity content in a Beckman gamma counter and time activity curves in percent per injected dose were generated for these organs. ⁶⁰Cu-ATSM and ⁶⁴Cu-ATSM organ residence times were calculated by numerical integration using the physical decay half-life of these nuclides from the decay corrected ⁶¹Cu-ATSM biodistribution data. Clearance by physical decay only was assumed for time points beyond the 4 hours post injection. Finally, human absorbed dose estimates were calculated according to the Medical Internal Radionuclide Dose (MIRD) methodology using S-value tables for ⁶⁰Cu (M.Stabin, private communication) and for ⁶⁴Cu (from MIRDose3.0). The absorbed dose to the bladder was calculated assuming no voiding and the dose to the organs was calculated assuming homogeneous distribution of the activity throughout the organs. All unmeasured activity, with the exception of excreted urine and feces, was assigned to the remainder of the body. Uptake in the lower intestinal track was assumed to be due to fecal matter and not due to hypoxia-related uptake. The detailed dosimetry procedure can be found in Laforest et al. (85). The estimated human dosimetry for both ⁶⁰Cu-ATSM and ⁶⁴Cu-ATSM is given in Table 2.
Table 2. Human radiation absorbed dose estimates for $^{60}$Cu-ATSM and $^{64}$Cu-ATSM extrapolated from $^{61}$Cu-ATSM rat biodistribution data.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Absorbed Dose for $^{60}$Cu-ATSM mSv/MBq (rem/mCi)</th>
<th>Absorbed Dose for $^{64}$Cu-ATSM * mSv/MBq (rem/mCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td>0.054 (0.198)</td>
<td>0.065 (0.239)</td>
</tr>
<tr>
<td>Bladder Wall</td>
<td>0.011 (0.040)</td>
<td>0.020 (0.074)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.019 (0.072)</td>
<td>0.053 (0.194)</td>
</tr>
<tr>
<td>Small Int.</td>
<td>0.043 (0.159)</td>
<td>0.095 (0.352)</td>
</tr>
<tr>
<td>ULI Wall</td>
<td>0.057 (0.211)</td>
<td>0.180 (0.668)</td>
</tr>
<tr>
<td>LLI Wall</td>
<td>0.043 (0.158)</td>
<td>0.775 (2.869)</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.083 (0.306)</td>
<td>0.497 (1.838)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.064 (0.238)</td>
<td>0.144 (0.532)</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.011 (0.039)</td>
<td>0.021 (0.079)</td>
</tr>
<tr>
<td>Marrow</td>
<td>0.010 (0.038)</td>
<td>0.017 (0.062)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.016 (0.059)</td>
<td>0.035 (0.130)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.007 (0.026)</td>
<td>0.011 (0.040)</td>
</tr>
<tr>
<td>Myocardium</td>
<td>0.013 (0.048)</td>
<td>0.041 (0.153)</td>
</tr>
<tr>
<td>Muscles</td>
<td>0.007 (0.027)</td>
<td>0.016 (0.061)</td>
</tr>
<tr>
<td>Bone Surfaces</td>
<td>0.009 (0.035)</td>
<td>0.010 (0.036)</td>
</tr>
<tr>
<td>Total Body</td>
<td>0.009 (0.035)</td>
<td>0.023 (0.087)</td>
</tr>
<tr>
<td>Effective Dose</td>
<td>0.020 (0.074)</td>
<td>0.063 (0.233)</td>
</tr>
</tbody>
</table>

* Unpublished data

7. CLINICAL EXPERIENCE

To date, investigators at Washington University have evaluated 124 cancer patients (25 NSCLC, 18 head and neck cancer, 41 cervical cancer, 13 breast cancer, 6 brain tumors; 5 primary and 1 metastatic NSCLC, and 21 rectal cancer) with $^{60}$Cu-ATSM. Images were evaluated qualitatively and quantitatively; and the investigators found heterogeneous uptake in these cancers. Most of these studies were performed with $^{60}$Cu-ATSM synthesized at Washington University under several different research protocols approved by the Radioactive Drug Research Committee at that institution in accordance with 21 CFR 361.1. A more recent
comparison study of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM was performed under the aegis of IND #62,675. The results of these studies are described below.

### 7.1. Pharmacokinetics and Product Metabolism in Humans

A subset of patients with lung cancer (n = 6, each underwent one study) and head and neck cancer (n = 3, each underwent 2 studies) underwent radial artery catheterization to allow rapid blood sampling during a dynamic $^{60}$Cu-ATSM PET study. With injection of $^{60}$Cu-ATSM and initiation of the dynamic PET scan sequence, 1.0-mL blood samples were obtained typically at 30-second intervals for the first 5 minutes, every minute for an additional 5 minutes and then at 20, 40 and 60 minutes post injection. A 0.25-mL aliquot of the blood sample was directly added to 0.5 mL n-octanol and vortexed for 30 seconds. This was performed in triplicate at each time point. The samples were then centrifuged at 5000 rpm for 5 minutes to cause complete partitioning of the aqueous from the octanol phase. The aqueous phase was then completely aspirated from the octanol phase. All fractions were counted on a Beckman 8000-gamma counter (Irvine, CA). The efficiency of this counter was previously determined for $^{60}$Cu. The efficiency of the extraction of $^{60}$Cu-ATSM with octanol was measured in each patient using a sample of the patient’s blood (prior to the radiotracer injection), adding a known quantity of $^{60}$Cu-ATSM ex vivo and processing as for the regular blood samples as described above. Furthermore, the assumption that the lipophilic radioactive species extracted into the octanol is intact $^{60}$Cu-ATSM was confirmed with thin-layer chromatography performed on the octanol fraction using silica gel TLC plates with ethyl acetate as the mobile phase. Radio-TLC detection was accomplished using a BIOSCAN System 200 Imaging Scanner (Washington, DC).

The blood data show two important features. First, rapid movement of $^{60}$Cu-ATSM into tissues occurs in the first 5 minutes. Second, $^{60}$Cu-ATSM blood levels are remarkably stable from 10 to 60 minutes. While there is undoubtedly ongoing metabolism/clearance of $^{60}$Cu-ATSM at this time, a pseudoequilibrium state is apparently maintained by slow $^{60}$Cu-ATSM movement back out of normoxic tissues into blood.

**Kinetic analysis:** Initial attempts at modeling included a classic three-compartment model identical to that used for FDG kinetic analysis. In this model, k3 represents the irreversible reduction of $^{60}$Cu-ATSM in the cell. Poor fitting of the kinetic data, large coefficients of variation in estimated parameters and occasional failures to converge complicated the parameter estimation. The k3 term could not be accurately estimated. Despite these problems, overall net uptake [$K = (K1*k3)/(k2+k3)$] was well behaved. Mean tumor K = 0.016/min (S.D. = 0.046) and mean muscle K = 0.0057/min (S.D. = 0.0061) showing tumors exhibited quite different behavior than normal muscle tissue. Importantly, it was observed that muscle activity was essentially constant after the first 10 minutes of the 60 minutes PET scan, and, furthermore, paralleled the blood $^{60}$Cu-ATSM time course. To allow evaluation of tumor trapping of $^{60}$Cu-ATSM without the need of blood activity these two observations have been used to simplify data collection and analysis. It can be shown that the estimate of net trapping of a radiotracer in a tumor (K) under conditions of blood activity of constant value, is merely the slope of the tumor activity divided by the blood activity. To avoid blood sampling, $^{60}$Cu-ATSM muscle activity is used as an estimate of blood activity. The tumor time-activity curve from 10 to 60 minutes first undergoes linear regression. To correct for differences in blood activity, the $^{60}$Cu-ATSM tumor slope index (%change/min) is then calculated as the tumor slope divided by the average muscle activity.

### 7.2. Safety

A total of 124 subjects have received $^{60}$Cu-ATSM. Ten of these subjects received $^{60}$Cu-ATSM twice within a period of one month (1 cervical cancer and 9 head and neck cancer). Eight subjects (3 breast cancer and 5 head and neck cancer) received $^{60}$Cu-ATSM three times over a
period as short as one week and as long as 8 weeks. The administered mass of Cu-ATSM was \( \leq 14 \mu g \). None of these subjects experienced any adverse effects or had any clinically detectable pharmacological effects. Since nearly all of these studies were performed in accordance with 21 CFR 361.1, safety assessments other than observation were not performed.

The only study performed to date under IND #62,675 was a crossover comparison study of \(^{60}\)Cu-ATSM and \(^{64}\)Cu-ATSM in 10 women with newly diagnosed squamous cell carcinoma of the uterine cervix. To test the safety of \(^{60}\)Cu-ATSM and \(^{64}\)Cu-ATSM, participants’ vital signs and various laboratory tests, including standard CBC (white blood cell counts, red blood cell counts, hemoglobin, hematocrit, platelets, neutrophils, lymphocytes, monocytes, eosinophils, basophils, bands) comprehensive metabolic panel (sodium, potassium, chloride, total CO\(_2\), glucose, creatinine, urea nitrogen, calcium, aspartate aminotransferase, alanine transaminase, alkaline phosphatase, total bilirubin, albumin) and urinalysis (specific gravity, PH, protein, glucose, ketones, bilirubin occult blood, urobilinogen, nitrites, leukocytes estrace), were monitored within 4 h before and 1 h to 7 days after radiopharmaceutical injection. All examinations were monitored for clinically significant changes that may have been related to Cu-ATSM administration. Changes > 20/min in the heart rate and > 20 mmHg in systolic and/or diastolic blood pressure were considered to be significant. Changes > 2 g/dL in hemoglobin, > 150 IU/L in alanine transaminase, > 0.5 mg/dL in total bilirubin, > 0.75 mg/dL in serum creatinine, etc. also considered to be significant. All patients had locally advanced cervical cancer with primary lesions >2.0 cm in diameter. The women ranged in age from 33 to 79 years; their clinical FIGO stages were IB1 in 1, IB2 in 1, IIIB in 3, IIB in 1 and IIIB in 4. The tumor histology was squamous cell carcinoma in all patients. Imaging with \(^{60}\)Cu-ATSM and \(^{64}\)Cu-ATSM was performed on separate days in a randomized order (4 patients underwent \(^{60}\)Cu-ATSM-PET first and 6 patients underwent \(^{64}\)Cu-ATSM-PET first). The time difference between the two scans averaged 5.8 days (range 1 - 9 days). If \(^{60}\)Cu-ATSM was injected first, \(^{64}\)Cu-ATSM was injected at least 24 hours (mean 3.5 days; range 1 - 7 days) after \(^{60}\)Cu-ATSM injection. If \(^{64}\)Cu-ATSM was injected first, \(^{60}\)Cu-ATSM was injected at least 6 days, i.e., at least 11 half-lives of \(^{64}\)Cu (mean 7.3 days; range 6 - 9 days) after \(^{64}\)Cu-ATSM injection.

There were no clinically significant changes in the vital signs or laboratory tests after injection of \(^{60}\)Cu-ATSM and \(^{64}\)Cu-ATSM in this crossover study. Two patients had changes in their blood cell counts following Cu-ATSM injection, which were felt to be related to the diagnosis of cervical cancer and/or sampling error rather than Cu-ATSM administration. No adverse events or clinically detectable pharmacological effects related to either \(^{60}\)Cu-ATSM or \(^{64}\)Cu-ATSM were observed.

7.3. Dosimetry

Whole-body imaging to evaluate human dosimetry has been performed on 5 subjects with \(^{60}\)Cu-ATSM. Three males and two females ranging in age from 56-63 years old were imaged at two time points after the injection of up to 13 mCi of \(^{60}\)Cu-ATSM. The average injected dose was 11.86 mCi. Whole-body imaging took place over 0-120 minutes after the injection using an ECAT HR+ (962) or ECAT EXACT (921) PET scanner (Siemens Medical Systems) at time points chosen to provide 10 evenly spaced biodistribution data points and to allow for combining the data from all 5 patients. Organ activity concentration was directly obtained by drawing regions of interest on the PET images for the following organs: the liver, kidneys and spleen. No other organ showed significant uptake of activity. Blood sample were taken at regular intervals from the subjects. The time-activity distribution of these organs was created by the compilation of the organ activity concentration corrected for decay of \(^{60}\)Cu and normalized by the injected activity from all 5 patients. The time-activity data were then fitted by a mathematical function made of the combination of exponentials, to represent the activity uptake followed by washout.
phases. Residence time was obtained by analytical integration of the fitted functions and included the decay of $^{60}\text{Cu}$ or $^{64}\text{Cu}$. $^{61}\text{Cu}$ is co-produced with a yield of 6.6% per unit dose of $^{64}\text{Cu}$ at the time of production. This radionuclide decays much quicker than $^{64}\text{Cu}$ and the radiation from radionuclidic impurity at the time of imaging is minimal. All unmeasured activity was assigned to the remainder of the body. Urine was collected from three patients and in all cases, the total activity collected accounted for less than 0.4% of the injected dose over the imaging period. No excretion was thus assumed, and all unmeasured activity was assigned to the remainder of the body.

Radiation dose estimates for $^{64}\text{Cu}$-ATSM to the standard adult male were calculated using the MIRD technique. The residence time was used with the standard adult male S-values calculated with MIRDOSE3. The radiation dose to each organ was computed as the sum of the self-dose and from the dose received from all other organs and from the remainder of the body. The detailed dosimetry procedure can be found in Laforest et al. (85). Table 3 lists human absorbed doses calculated from $^{60}\text{Cu}$-ATSM-PET imaging in human and extrapolated to an administered activity of 25 mCi (925 MBq) of $^{64}\text{Cu}$-ATSM.

### Table 3: Human absorbed dose estimates from intra-venous injection of $^{64}\text{Cu}$-ATSM.

<table>
<thead>
<tr>
<th>Organ</th>
<th>mSv/MBq (rem/mCi)</th>
<th>mSv/925 MBq (rem/25 mCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td>0.032 (0.118)</td>
<td>29.5 (2.95)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.013 (0.047)</td>
<td>11.8 (1.18)</td>
</tr>
<tr>
<td>Breast</td>
<td>0.016 (0.058)</td>
<td>14.5 (1.45)</td>
</tr>
<tr>
<td>Gallbladder wall</td>
<td>0.068 (0.252)</td>
<td>63.0 (6.30)</td>
</tr>
<tr>
<td>LLI Wall</td>
<td>0.022 (0.081)</td>
<td>20.2 (2.02)</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>0.019 (0.072)</td>
<td>18.0 (1.80)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.021 (0.078)</td>
<td>19.2 (1.92)</td>
</tr>
<tr>
<td>ULI Wall</td>
<td>0.022 (0.080)</td>
<td>20.0 (2.00)</td>
</tr>
<tr>
<td>Heart Wall</td>
<td>0.029 (0.107)</td>
<td>26.8 (2.68)</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.088 (0.326)</td>
<td>81.2 (8.12)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.390 (1.443)</td>
<td>360.8 (36.08)</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.021 (0.078)</td>
<td>19.5 (1.95)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.017 (0.062)</td>
<td>15.5 (1.55)</td>
</tr>
<tr>
<td>Ovaries</td>
<td>0.017 (0.062)</td>
<td>15.5 (1.55)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.056 (0.207)</td>
<td>51.8 (5.18)</td>
</tr>
<tr>
<td>Red Marrow</td>
<td>0.019 (0.071)</td>
<td>17.8 (1.78)</td>
</tr>
<tr>
<td>Bone Surfaces</td>
<td>0.015 (0.057)</td>
<td>14.2 (1.42)</td>
</tr>
<tr>
<td>Skin</td>
<td>0.014 (0.050)</td>
<td>12.5 (1.25)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.047 (0.174)</td>
<td>43.8 (4.38)</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.017 (0.062)</td>
<td>15.5 (1.55)</td>
</tr>
<tr>
<td>Urinary Bladder Wall</td>
<td>0.015 (0.055)</td>
<td>13.8 (1.38)</td>
</tr>
<tr>
<td>Uterus</td>
<td>0.017 (0.062)</td>
<td>15.5 (1.55)</td>
</tr>
<tr>
<td>Total Body</td>
<td>0.026 (0.098)</td>
<td>24.5 (2.45)</td>
</tr>
<tr>
<td>Effective Dose (ED)*</td>
<td>0.039 (0.144)</td>
<td>36.0 (3.60)</td>
</tr>
<tr>
<td>Effective Dose Equivalent (EDE)*</td>
<td>0.049 (0.182)</td>
<td>45.5 (4.55)</td>
</tr>
</tbody>
</table>

* Calculated for females only
7.4. Efficacy

Patients with several types of cancers have been studied with $^{60}$Cu-ATSM-PET at Washington University. All patients gave written informed consent prior to participating in the study. All imaging was performed utilizing either a Siemens ECAT Exact (Siemens-CTI, Knoxville, TN) or a Siemens ECAT EXACT HR+ (Siemens-CTI, Knoxville, TN) PET scanner. All patients underwent a dynamic study following administration of $^{60}$Cu-ATSM for a total of 60 minutes. All patients also underwent a 15-minute transmission scan utilizing $^{68}$Ge/$^{68}$Ga rod sources prior to administration of $^{60}$Cu-ATSM. $^{60}$Cu-ATSM-PET images were reconstructed by filtered back-projection using measured coefficients calculated from transmission scans, with a Hanning filter (frequency cutoff approximately 0.3 cycles/pixel) and smoothing to approximately 8 mm (full width at half maximum). All $^{60}$Cu-ATSM-PET images were evaluated qualitatively by an experienced nuclear medicine physician and correlated with FDG-PET (when available) and computed tomography (CT) images. Regions of interest (ROIs) were drawn in multiple planes, on the 30-60 summed images, guided by CT (and clinical FDG-PET when available), in order to create volumes of interest (VOIs) (using ANALYZE, Mayo Clinic). Similar VOIs were drawn on the known tumor cancer and bilateral back (or gluteal muscles in cervical and rectal cancers and neck muscles for head and neck cancer) muscle groups. The overall tumor uptake of $^{60}$Cu-ATSM was assessed semiquantitatively on the 30-60 minute summed images by determining tumor-to-muscle activity ratio (T/M) using maximum-pixel value for the tumor and the average value for muscle. This interval was based on our previous studies in patients with NSCLC and cervical cancer; these demonstrated that the semiquantitative analysis of the summed data from 30 to 60 min post injection provides information similar to that achieved by a more formal quantitative analysis of the images (3, 5, 6). In addition, this time interval represented a practical tradeoff between image contrast and image noise, considering the short half-life of $^{60}$Cu-ATSM. In order to depict the rate of $^{60}$Cu-ATSM trapping in selected patients, a parametric image was created by calculating the pixel-by-pixel slope (tissue activity vs. time) using decay-corrected images from 10 to 60 minutes. To normalize the slope data, an image of mean $^{60}$Cu-ATSM activity during this same time frame was created as well. A peak slope index (%/min) was calculated from the tumor VOI on the slope image by dividing the peak slope seen within the entire tumor VOI by the mean muscle activity. No significant difference was noted between the results of parametric (peak slope index) and semiquantitative analysis (T/M) of the images in patients whose images were analyzed by these two methods; thus, the simpler semiquantitative method was used for analysis of images. In addition, $^{60}$Cu-ATSM images were evaluated using maximum standardized uptake value (SUV$_{max}$) in a subset of the patients. However, the tumor SUV for $^{60}$Cu-ATSM did not correlate with response to therapy or overall survival in patients with lung and cervical cancers (see below). While SUV has been widely used as a reliable and practical method for semiquantitative analysis of FDG images in oncologic patients, the SUV has rarely been used in human studies of tracers other than FDG. In the case of $^{60}$Cu-ATSM there may be too much intersubject variability in the clearance or distribution of the tracer for SUV to be an effective measure.

A brief description of results of $^{60}$Cu-ATSM–PET studies in the cancers studied are as follows:

7.4.1. Head and Neck Cancer Study

In a pilot study to assess the kinetics of $^{60}$Cu-ATSM uptake with PET, 18 patients with T2-T4 head and neck carcinoma were studied prospectively. Fifteen males and 3 females ranging in age from 49-73 years of age underwent a total of 37 examinations (3 underwent one study, 9 underwent 2 studies and 5 underwent 3 studies) with $^{60}$Cu-ATSM. Injected dose for this group of subjects ranged from 9 to 13 mCi of $^{60}$Cu-ATSM. Supine imaging with the patient's treatment thermoplastic immobilization head mask and
fiducial markers was performed at baseline, and after approximately 20 and 40 Gy of therapy. These studies helped in understanding the ⁶⁴Cu-ATSM kinetics (See 7.3.2. for details). In addition in patients with sufficient clinical follow-up (n =12), it was found that patients with T/M > 4.0 had poorer prognosis than patients with T/M ≤ 4.0 (unpublished data). We were able to co-register ⁶⁴Cu-ATSM and CT images using the thermoplastic immobilization head mask to create a hypoxia imaging-guided IMRT treatment plan for radiation therapy of patients with head and neck cancer (7).

7.4.2. Non-Small Cell Lung Cancer Study
This study prospectively evaluated 25 patients (mean age 68 years, range 55 - 84 years) with suspected or proven stages stage II-IV NSCLC (1 of the 25 patients later proved to have a benign lung nodule and 4 of the 25 patients did not complete imaging session and thus their imaging data were not included in the final analysis). Each patient had at least one lesion greater ≥1.5 cm. Six patients did not complete the imaging and their data were not analyzed. In patients with multiple lesions, only the largest lesion was chosen for analysis. Five subjects, three males and two females underwent dosimetry imaging as part of this pilot study as described in section 7.3. Injected dose for this group of subjects ranged from 2.3 to 13 mCi of ⁶⁴Cu-ATSM. Injected dose in the remaining patients ranged from 6 to 13.0 mCi of ⁶⁴Cu-ATSM. The tumor of one patient had no discernible ⁶⁴Cu-ATSM uptake, whereas the tumor uptake in the remaining patients was variable, as expected. Response was evaluated in 14 patients; the mean T/M for ⁶⁴Cu-ATSM was significantly lower in responders (1.5 ± 0.4) than in nonresponders (3.4 ± 0.8) (P=0.002). However, the mean SUV for ⁶⁴Cu-ATSM was not significantly different in responders (2.8 ± 1.1) and nonresponders (3.5 ± 1.0) (P = 0.2). An arbitrarily selected T/M threshold of 3.0 discriminated those likely to respond to therapy: all eight responders had a T/M < 3.0 and all six nonresponders had a T/M > or =3.0. These patients also underwent clinical FDG-PET. Tumor SUV for FDG was not significantly different in responders and nonresponders (P = 0.7) and did not correlate with ⁶⁴Cu-ATSM uptake (r = 0.04; P = 0.9). Thus, it was concluded that ⁶⁴Cu-ATSM-PET can be readily performed in patients with NSCLC and the tumor uptake of ⁶⁴Cu-ATSM reveals clinically unique information about tumor oxygenation that is predictive of tumor response to therapy (6).

Kinetic analysis in subset of patients with head and neck and NSCLC: Initial attempts at modeling included a classic three-compartment model identical to that used for FDG kinetic analysis. In this model, k₃ represents the irreversible reduction of ⁶⁴Cu-ATSM in the cell. Poor fitting of the kinetic data, large coefficients of variation in estimated parameters and occasional failures to converge complicated the parameter estimation. The k₃ term could not be accurately estimated. Despite these problems, overall net uptake [K = (K₁*k₃)/(k₂+k₃)] was well behaved. Mean tumor K = 0.016/min (S.D. = 0.046) and mean muscle K = 0.0057/min (S.D. = 0.0061) showing tumors exhibited quite different behavior than normal muscle tissue. Importantly, it was observed that muscle activity was essentially constant after the first 10 minutes of the 60 minutes PET scan, and, furthermore, paralleled the blood ⁶⁴Cu-ATSM time course. To allow evaluation of tumor trapping of ⁶⁴Cu-ATSM without the need of blood activity these two observations were used to simplify data collection and analysis. It can be shown that the estimate of net trapping of a radiotracer in a tumor (K) under conditions of blood activity of constant value, is merely the slope of the tumor activity divided by the blood activity. To avoid blood sampling, ⁶⁴Cu-ATSM muscle activity was used as an estimate of blood activity. To correct for differences in blood activity, the ⁶⁴Cu-ATSM tumor slope index (%change/min) was then calculated as the tumor slope divided by the average muscle
activity. This method was compared with T/M ratio in these patients and in 27 of 38 patients with cervical cancer and no significant difference was found between these two methods.

Lohith et al. recently demonstrated that the intratumoral distribution patterns of $^{62}$Cu-ATSM and FDG may be dependent on tumor histology in patients with lung cancer (86). They studied 13 patients (8 with lung cancer squamous cell carcinoma and 5 with adenocarcinoma) with $^{62}$Cu-ATSM-PET and FDG-PET. $^{62}$Cu-ATSM-PET and FDG-PET images were coregistered, and multiple small regions of interest were drawn on tumor lesions of the 2 images for measurement of SUV. The regression lines were determined between SUVs for tumor uptake of $^{62}$Cu-ATSM and FDG. The slope values were compared between squamous cell carcinoma and adenocarcinoma to observe pathohistologic differences in intratumoral distribution of the tracers. They found that SUVs for $^{62}$Cu-ATSM were lower than those for FDG in both squamous cell carcinoma and adenocarcinoma. Tumors with squamous cell carcinoma histology showed high $^{62}$Cu-ATSM and low FDG uptake in the peripheral region of the tumor but low $^{62}$Cu-ATSM and high FDG uptake toward the center (spatial mismatching). The relationship of SUV for the 2 tracers was negatively correlated with a mean regression slope of $-0.07 \pm 0.05$. Tumors with adenocarcinoma histology had a spatially similar distribution of $^{62}$Cu-ATSM and FDG, with positive regression slopes averaging $0.24 \pm 0.13$. The regression slopes for $^{62}$Cu-ATSM and FDG differed significantly between SCC and adenocarcinoma ($P < 0.001$).

7.4.3. Rectal Cancer Study
This was a prospective study of 21 patients (15 males, 6 females, ranging 32-71 yrs) with T2-T4 rectal carcinoma prior to neoadjuvant chemoradiotherapy. The administered dose was 7 to 13 mCi of $^{60}$Cu-ATSM. The data of 4 patients were not included in the final analysis (2 did not complete imaging, 2 did not complete therapy). Of the 17 remaining patients, 14 had a reduction in tumor size and 13 were down staged. The median T/M ratio of 2.6 discriminated those with worse prognosis from those with better prognosis. Both overall and progression-free survivals were worse with hypoxic tumors (T/M > 2.6) than with non-hypoxic tumors (T/M ≤ 2.6) (both p< 0.05). In addition, 2 of the 3 tumors with no change in size had T/M > 2.6 [positive predictive value (PPV) 66%] while 6/14 with decreased size had T/M > 2.6 [negative predictive value (NPV) 57%]. Three of the 4 tumors not down staged had T/M > 2.6 (PPV 75%) while 5/13 down staged tumors had T/M > 2.6 (NPV 62%). The mean T/M ratio for down staged tumors (2.2) was significantly lower than that of non-down staged tumors (3.3) (p = 0.03). The difference in mean T/M ratio between downsized (2.3) and non-downsized (2.9) tumors did not reach statistical significance (p = 0.36). Tumor FDG uptake (n =11) did not correlate with $^{60}$Cu-ATSM uptake (r = 0.4; p = 0.9) and there was no significant difference in mean tumor FDG uptake between patients with hypoxic tumors and those with normoxic tumors (p = 0.3) (5).

7.4.4. Initial Cervical Cancer Study
Initially, a prospective study of $^{60}$Cu-ATSM-PET was performed in14 patients (aged 23-84 years) with cervical cancer before initiation of radiotherapy and chemotherapy. All patients had locally advanced cervical cancer with primary lesions > 2.0 cm in diameter (FIGO clinical stage IB1 in 1, stage IB2 in 1, stage IIB in 8, and stage IIB in 4). The tumor histology was squamous cell carcinoma in 13 and adenosquamous carcinoma in 1. All patients also underwent clinical FDG-PET prior to therapy. The PET results were correlated with follow-up evaluation (14 to 24 months). All patients received external
irradiation and intracavitary brachytherapy. Concurrent weekly cisplatin chemotherapy (40 mg/m² weekly for six cycles) was given to 13 patients; the remaining patient did not receive chemotherapy (because of severe co-morbidity). Tumor uptake of ⁶⁰Cu-ATSM was inversely related to progression-free survival and overall survival (log-rank, p = 0.0005 and p = 0.015, respectively). An arbitrarily selected T/M threshold of 3.5 discriminated those likely to develop recurrence; 6 (T/M < 3.5) were free of disease at last follow-up whereas all of 5 patients with hypoxic tumors (T/M > 3.5) had already developed recurrence. Similar discrimination was achieved with the peak slope index. The frequency of locoregional nodal metastasis was greater in hypoxic tumors (p = 0.03). Tumor FDG uptake did not correlate with ⁶⁰Cu-ATSM uptake (r = 0.04; p = 0.80) and there was no significant difference in tumor FDG uptake between patients with hypoxic tumors and those with normoxic tumors (4).

7.4.5. Expanded Cervical Cancer Study

The work in cervical cancer was extended in a prospective study of a total of 38 patients (aged 23-84 years) with biopsy-proved cervical cancer who underwent ⁶⁰Cu-ATSM-PET before initiation of therapy. All patients had locally advanced cervical cancer with primary lesions > 2.0 cm in diameter (FIGO clinical stage Ib1 in 3, stage Ib2 in 3, stage IIa in 1, stage IIb in 18, IIIa in 1, IIIb in 11, IVa in 1). The tumor histology was squamous cell carcinoma in 37 and adenosquamous carcinoma in 1. The results for 14 of these patients included in the pilot study have been reported previously (4). The brief description of these results can be found in section 7.4.4. All patients were initially evaluated before treatment with a history (smoking history was not recorded) and physical examination, routine laboratory studies (all patients had hemoglobin levels measured and these averaged 11.9 ± 2.4 g/dL prior to therapy), cervical biopsy, chest radiography, abdominopelvic CT, and clinical whole-body FDG-PET, performed as part of our clinical routine. All patients received external irradiation and intracavitary brachytherapy. Concurrent weekly cisplatin chemotherapy (40 mg/m² weekly for six cycles) was given to 35 patients; the remaining 3 patient did not receive chemotherapy (because of severe co-morbidity). Clinical follow-up was performed six weeks after completion of radiotherapy and at 3-month intervals for the next 2 years and then every 6 months thereafter. The duration of follow-up for patients alive at the time of last evaluation ranged from 3 to 79 months. Progression-free survival and cause-specific survival were measured from the date of completion of irradiation to the date of recurrence or death. To assess whether the tumor uptake of either ⁶⁰Cu-ATSM or FDG is predictive of treatment response, the PET results were correlated with the results of clinical follow-up. The physician who assessed the patients for disease progression was blinded to the results of the ⁶⁰Cu-ATSM studies. The Kaplan-Meier method was used to assess the relationship between ⁶⁰Cu-ATSM uptake and both progression-free and cause-specific survival rates. Equivalence of survival curves was tested with the log-rank (Mantel-Cox) statistics. A log rank test was used to determine the cut-off uptake value that was strongly predictive of prognosis. The relationships between ⁶⁰Cu-ATSM uptake in the primary tumor and the presence of metastatic involvement of pelvic lymph nodes (as assessed by FDG-PET) and stage of disease were assessed by the Fisher's exact and the chi square statistics, respectively. The correlation between tumor uptake of ⁶⁰Cu-ATSM and that of FDG was evaluated by linear regression (3).

At last follow-up, 27 patients were alive for periods ranging from 3 to 84 months (median 41 months), 24 with no evidence of cervical cancer and 3 with a recurrence of cervical cancer. The remaining 11 had died, 10 due to recurrent cervical cancer and 1 due to intercurrent disease). Using log-rank analysis of our previously reported data, it was
found that a T/M threshold of 3.5 was a statistically significant cut-off value that accurately distinguished patients whose cancer did not recur from those who developed a recurrence after completing therapy. In the current study, this cut-off value appeared to have similar discriminatory power. The Kaplan-Meier survival estimates for patients with T/M above and below 3.5 are shown in Figure 2a and 2b. Progression-free and cause-specific survival were significantly better in patients with T/M above 3.5 (p = 0.006 and p = 0.04, respectively). The 3-year progression-free survival of patients with normoxic tumors (T/M ≤ 3.5) was 71% and that of patients with hypoxic tumors (T/M > 3.5) was 28% (p = 0.01) (Figure 3). The corresponding cause-specific survival estimates were 74% and 49%, respectively (p = 0.05). There was no significant difference in the frequency of lymph node involvement between patients with T/M above (9/16; 56%) and below 3.5 (9/22; 41%) (p = 0.6). Also, there was no significant correlation between disease stage and tumor uptake of $^{64}$Cu-ATSM (p = 0.46). There was no significant difference in total radiation dose (p = 0.22) or overall treatment time (p = 0.98) between patients with T/M above or below 3.5. There was no significant correlation between tumor SUV$_{\text{max}}$ for FDG and tumor uptake of $^{64}$Cu-ATSM (R$^2 = 0.006$, p = 0.63). The mean tumor FDG uptake in hypoxic tumors was 11.7 ± 4.2 (p = 0.9 by unpaired t-test) and in those with normoxic tumors was 11.5 ± 8.4 (p = 0.9 by unpaired t-test) (3).
Figure 2a. Hypoxic tumor. Sagittal FDG-PET/CT image (right) of the pelvis shows intense FDG uptake in the known primary cervical cancer. Sagittal $^{60}$Cu-ATSM-PET image co-registered with CT image (left) at the same level also demonstrates intense uptake within the primary cervical cancer ($T/M = 4.5$). Note that there are different patterns of FDG and $^{60}$Cu-ATSM uptake within the primary tumor. $P =$ primary tumor; $B =$ urinary bladder. Adapted from *J Nucl Med.* 2008;49:201-205.

Figure 2b. Normoxic Tumor. Sagittal FDG-PET/CT image (right) of the pelvis shows intense FDG uptake in the known primary cervical cancer. Sagittal $^{60}$Cu-ATSM-PET image co-registered with CT image (left) at the same level demonstrates only mildly increased uptake of this tracer within the primary cervical cancer ($T/M = 3.0$). As in Figure 3, there are different patterns of FDG and $^{60}$Cu-ATSM uptake within the primary tumor. $P =$ primary tumor; $B =$ urinary bladder. Adopted from *J Nucl Med.* 2008;49:201-205.

Figure 3. Progression-free survival (left) and cause-specific survival (right) based on $^{60}$Cu-ATSM uptake using Kaplan-Meier method. ($\circ =$ event in patients with $T/M \leq 3.5$ and $\triangledown =$ event in patients with $T/M > 3.5$). Adopted from *J Nucl Med.* 2008;49:201-205.
The initial Washington University studies were performed using $^{60}$Cu-labeled ATSM, which was suitable for local production; however, for radiopharmaceutical distribution to multiple centers, longer-lived $^{64}$Cu is desirable. The longer half-life of $^{64}$Cu (12.7 hours) would allow shipment from a single production site to other US imaging facilities, thus making hypoxia imaging more widely available. In addition, the lower positron energy for $^{64}$Cu than for $^{60}$Cu reduces image blurring and leads to better spatial resolution.

### 7.4.6. Cervical Cancer Crossover Comparison Study

The crossover comparison study of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM performed under IND #62,675 included 10 women with newly diagnosed squamous cell carcinoma of the uterine cervix, who were evaluated before beginning conventional treatment by chemoradiotherapy. All patients had locally advanced cervical cancer with primary lesions $>2.0$ cm in diameter. The women ranged in age from 33 to 79 years; their clinical FIGO stages were IB1 in 1, IB2 in 1, IIIB in 3, IIIA in 1 and IIIB in 4. The tumor histology was squamous cell carcinoma in all patients. All patients gave written informed consent prior to participating in the study. PET imaging with $^{60}$Cu-ATSM and $^{64}$Cu-ATSM was performed with a CTI/Siemens ECAT HR+ scanner (Siemens-CTI, Knoxville, TN). Imaging with $^{60}$Cu-ATSM and $^{64}$Cu-ATSM was performed on separate days in a randomized order (4 patients underwent $^{60}$Cu-ATSM-PET first and 6 patients underwent $^{64}$Cu-ATSM-PET first). The time difference between the two scans averaged 5.8 days (range 1 - 9 days). If $^{60}$Cu-ATSM was injected first, $^{64}$Cu-ATSM was injected at least 24 hours (mean 3.5 days; range 1 - 7 days) after $^{60}$Cu-ATSM injection. If $^{64}$Cu-ATSM was injected first, $^{60}$Cu-ATSM was injected at least 6 days, i.e., at least 11 half-lives of $^{64}$Cu (mean 7.3 days; range 6 - 9 days) after $^{64}$Cu-ATSM injection. In order to optimize image quality, all 10 patients were to receive approximately 925 MBq (25 mCi) of $^{64}$Cu-ATSM and 740 MBq (20 mCi) $^{60}$Cu-ATSM. These dosages of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM were chosen to acquire the same number of decays in the 30- to 60-min post-injection scan. A 925 MBq (25 mCi) dosage of $^{64}$Cu-ATSM results in a 361 mSv dose to the liver and an effective dose of 33.3 mSv, the corresponding doses from a 740 MBq dose of $^{60}$Cu-ATSM are 47.4 mSv and 8.14 mSv ($^8$).

The imaging procedure and image processing were the same for both $^{60}$Cu-ATSM-PET and $^{64}$Cu-ATSM-PET except with regard to imaging mode and the treatment of the cascade gamma rays emission. The $^{64}$Cu-ATSM-PET images were acquired in the 3D mode (with septa retracted) to maximize sensitivity. As for the phantom studies, all imaging with $^{60}$Cu was performed in the 2D mode, since the collimator septa minimize contamination by cascade coincidences. Where the injected activity of $^{60}$Cu-ATSM was less than the planned 740 MBq (20 mCi), the duration of image acquisition with $^{64}$Cu-ATSM was adjusted so that the images with both radionuclides had similar count statistics. Since the data from 30 to 60 min were acquired as six 5-min frames, the summed image was created from the approximate number of 5-min frames to achieve matching image statistics.

Given that $^{60}$Cu decays by positron decay with the concurrent emission of numerous cascade gamma photons, the fortuitous cascade coincidences were removed by convolution of the cascade gamma ray kernel. Using this technique, the fully corrected projection data (corrected for normalization, attenuation and scatter) were further corrected by subtraction of the cascade background and the images were then reconstructed from those corrected data with the same reconstruction algorithm ($^8$). All imaging with $^{60}$Cu was performed in 2D mode since the collimator septa minimize contamination by cascade coincidences.
For qualitative analysis, the $^{60}$Cu-ATSM-PET and $^{64}$Cu-ATSM-PET images were evaluated subjectively by an experienced nuclear medicine physician who was blinded as to which Cu-ATSM scan was being interpreted. Then the images were evaluated in correlation with the CT and FDG-PET/CT images. In addition, the overall tumor uptake of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM was assessed semiquantitatively by determining T/M based on the 30-to-60 min summed images. For the $^{60}$Cu-ATSM images, the patient data were processed by the cascade subtraction technique described above.

The 10 patients received a mean of 903 MBq (24.3 mCi) (range 821 - 952 MBq) of $^{64}$Cu-ATSM and a mean of 478 MBq (12.9 mCi) (range 204 - 740 MBq of $^{60}$Cu-ATSM. Increased $^{60}$Cu-ATSM and $^{64}$Cu-ATSM uptake was seen in the tumors of all 10 patients (T/M of 5.9 ± 1.6 and 7.4 ± 1.9, respectively). A significant correlation was observed between the uptake of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM ($r = 0.95, P <0.0001$) (8). The image quality was comparable; although generally, the images with $^{64}$Cu-ATSM had a slightly better target-to-background ratio and tumors were delineated more clearly by comparison with the $^{60}$Cu-ATSM images (Figure 4). Importantly, the pattern of uptake was similar on the images obtained with the tracers during two different imaging sessions 1 to 9 days apart, indicating that the macroscopic distribution of hypoxia did not change greatly over this interval.

![Figure 4](image-url)

**Figure 4** (A) Transaxial CT (top left) and FDG-PET (top right) images of the pelvis show intense FDG uptake within the known cervical tumor at the site of the cervical mass seen on CT. Transaxial 30-60 min summed images of $^{60}$Cu-ATSM-PET (lower left) and $^{64}$Cu-ATSM-PET (lower right) of the pelvis at the same level demonstrates mildly increased uptake within the known primary cervical tumor. There are similar patterns of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM uptake within the tumor. (B) Transaxial co-registered FDG-PET/CT (top left) and FDG-PET (top right) images of the pelvis show intense FDG uptake within the known cervical tumor at the site of the cervical mass seen on CT. Transaxial 30-60 min summed images of $^{60}$Cu-ATSM-PET (lower left) and $^{64}$Cu-ATSM-PET (lower right) of the pelvis at the same level demonstrates markedly increased uptake within the known primary cervical tumor. There are similar patterns of $^{60}$Cu-ATSM and $^{64}$Cu-ATSM uptake within the tumor. Adopted from *J Nucl Med.* 2008; 49:1177-82.

Given that $^{60}$Cu decays by positron decay with the concurrent emission of numerous cascade gamma photons, the fortuitous cascade coincidences were removed by
convolution of the cascade gamma ray kernel as previously reported and validated for 
\(^{86}\text{Y}\) and \(^{76}\text{Br}\) imaging in clinical cameras (87, 88). Using this technique, the fully corrected 
projection data (corrected for normalization, attenuation and scatter) were further 
corrected by subtraction of the cascade background and the images were then 
reconstructed from those corrected data with the same reconstruction algorithm. The 
\(^{64}\text{Cu}\)-ATSM PET images were acquired in the 3D mode (with septa retracted) to 
maximize sensitivity. The imaging with \(^{60}\text{Cu}\) was performed in 2D mode since the 
collimator septa minimize contamination by cascade coincidences. The patient data 
also were quantitatively analyzed and T/M ratios were measured on the images obtained 
with \(^{64}\text{Cu}\)-ATSM, \(^{60}\text{Cu}\)-ATSM, and \(^{60}\text{Cu}\)-ATSM processed with cascade subtraction. Note that correction of the \(^{60}\text{Cu}\)-ATSM data for coincidences attributable to the gamma 
photon cascade emitted when this radionuclide decays led to T/M ratios more similar to 
those obtained with \(^{64}\text{Cu}\)-ATSM; the mean T/M for the corrected \(^{60}\text{Cu}\)-ATSM data was 
7.3 ± 1.8, closely similar to those reported above for \(^{64}\text{Cu}\)-ATSM. Additionally, the slope 
of the regression line was 1.002 with the corrected \(^{60}\text{Cu}\)-ATSM values versus 1.238 
without the cascade subtraction correction. The corresponding correlation coefficients 
were 0.88 and 0.95, respectively (8).

In addition, the patient data were quantitatively analyzed and T/M ratios were measured 
on the images obtained with \(^{64}\text{Cu}\)-ATSM, \(^{60}\text{Cu}\)-ATSM, and \(^{60}\text{Cu}\)-ATSM processed with 
cascade subtraction. This analysis demonstrated that similar T/M ratios are obtained 
with both radionuclides and that application of the cascade subtraction correction 
improves the correlation between the \(^{64}\text{Cu}\)-ATSM and the \(^{60}\text{Cu}\)-ATSM measurements. The T/M for \(^{60}\text{Cu}\)-ATSM processed with cascade subtraction was 7.3 ± 1.8, closely 
similar to that reported above for \(^{64}\text{Cu}\)-ATSM (7.4 ± 1.9). The slope of the regression line 
was 1.002 with the corrected \(^{60}\text{Cu}\)-ATSM values versus 1.238 without the cascade 
subtraction correction. The corresponding correlation coefficients were 0.88 and 0.95, 
respectively (8).

**Figure 5.** Correlation of \(^{60}\text{Cu}\)-ATSM uptake (without and with cascade 
subtraction [CS]) and \(^{64}\text{Cu}\)-ATSM uptake in 10 patients with cervical 
cancer. Results are expressed as 
T/M ratios. Good correlation 
between uptakes of these 2 
radiotracers was found. This 
analysis was performed to 
demonstrate that similar T/M ratios 
can be obtained with both nuclides 
and that applying \(^{60}\text{Cu}\) cascade 
coincidence correction improves 
comparability of measured T/M 
ratios. Linear regressions were 
determined by setting y-intercept to 
zero and slope derived from least-
square minimization. Adopted from 
8. SUMMARY OF DATA AND GUIDANCE FOR THE INVESTIGATOR

The preclinical and clinical data obtained with $^{60}$Cu-ATSM and $^{64}$Cu-ATSM indicate that the latter appears to be a safe radiopharmaceutical that can be used to obtain high quality images of tumor hypoxia in human cancers.

8.1. Adverse Events
An Adverse Event (AE) is any untoward medical occurrence in a participant that does not necessarily have a causal relationship with the study procedure. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory or physiological finding), symptom, or disease temporally associated with the use of a medical treatment or procedure, regardless of whether it is considered related to the medical treatment or procedure (attribution of unrelated, unlikely, possible, probable, or definite).

Based on experience to date, no adverse events directly attributable to $^{64}$Cu-ATSM are expected.

8.2. Drug Interactions
None known.

8.3. Immunogenicity
None known.

8.4. Carcinogenesis, Mutagenesis, Impairment of Fertility
None known.

8.5. Special Population
Although not specifically studied to date, there are no available data to suggest that the safety or effectiveness of $^{64}$Cu-ATSM will vary as a function of age, gender, race, or ethnicity. $^{64}$Cu-ATSM is not intended for use in children.

8.6. Overdose
Not applicable.
9. REFERENCES


39. Tannock I, Guttmann P. Response of Chinese hamster ovary cells to anticancer drugs


66. Fujibayashi Y, Taniuchi H, Yonekura Y, Ohtani H, Konishi J, Yokoyama A. Copper-62-


10. Appendices

10.1 Appendix 1

Preparation of $^{64}$Cu-ATSM using PTI Kit Formulation (Version 2) (Preferred Method)
10.1 Appendix 1

**Preparation of $^{64}\text{Cu-ATSM}$ via PTI Kit Formulation (Version 2)**

**Index:**
- I. Overview
- II. Training
- III. Equipment
- IV. Procedure

I. Overview

This document describes the procedure for the Version 2 preparation of $^{64}\text{Cu-ATSM}$ using the Proportional Technologies, Inc. (PTI) kit.

II. Training

This procedure can only be carried out by trained personnel under the supervision of the Authorized User (AU)/Principal Investigator (PI)/Authorized Nuclear Pharmacist (ANP) or his/her designees. A list of trained personnel will be kept by the AU/PI/ANP and available upon request. Personnel must understand and practice all relevant safety and security procedures.

III. Equipment

A. Equipment

- Dose Calibrator
- Vortex
- Two Lead Shields for 10-mL Vials
- Tongs

B. Supplies

- Proportional Technologies, Inc. (PTI) Kit (consisting of Reconstitution Solution in 10-mL vial and Lyophilized ATSM Ligand in 50-mL Vial)
- $^{64}\text{CuCl}_2$ solution in 0.1 M HCl (Supplied by Washington University in a 10-mL vial)
- Three 0.22-µm Millex-GV Sterilizing Filter Units (Millipore) (or equivalent)
- Three 10-mL Luer-Lok Syringes (Terumo SS-10L or equivalent)
- Five 21-gauge Needles (Kendal Monoject 1188 or equivalent)
- One 19 gauge 3 ½” Spinal Needle (B/D 405183 or equivalent)
- One 33” Extension Tubing (Smiths Medical PORMX455FL or equivalent)
- One 6” Extension Tubing (Smiths Medical MX452 or equivalent)
- One 10 mL Sterile Empty Vial (Hospira ABB5816-11 or equivalent)
- Six Sterile 70% Isopropyl Alcohol (IPA) Pads (Kendall 6818 or equivalent)
IV. Procedure

A. Personal Protection

1. All personnel must don lab coats, eye protection, and disposable protective shoe covers before entering the laboratory.
2. Sleeve protectors and two pair of disposable gloves must be worn during the preparation.
3. All personnel must wear TLD radiation badges and rings.

B. Drug Compounding

1. It is recommended that radiopharmaceutical compounding proceed in a Class 100 environment
2. Drug preparation should involve aseptic processing
3. Principles of ALARA must be observed for all aspects of drug compounding.

C. Reconstitution of ATSM Ligand

1. Swab the septa of the two PTI kit vials with sterile IPA pads.
2. Attach a 21-gauge needle to a 10-mL syringe.
3. Withdraw 10 mL of the reconstitution solution into the 10-mL syringe.
4. Discard the empty syringe-needle assembly appropriately.
5. Inject the contents of the 10-mL syringe into the 50-mL vial containing the lyophilized ATSM ligand.
6. Grasp the lip of the 50-mL vial with the tongs, and mix the solution vigorously for at least one minute with the vortex.

D. Radiolabeling of ATSM Ligand

1. Using a sterile IPA pad, swab the septum of the 50-mL vial containing the reconstituted ATSM ligand.
2. Using tongs and a sterile IPA pad, swab the septum of the 10 mL $^{64}$CuCl$_2$ vial in the lead shield.
3. Attach a 21-gauge needle to a 10-mL syringe.
4. Withdraw the reconstituted ATSM ligand solution into the 10-mL syringe.
5. Inject the contents of the 10-mL syringe into the shielded 10-mL vial containing $^{64}$CuCl$_2$. This is the Reaction Vial.
6. Discard the empty syringe-needle assembly appropriately.
7. Grasp the lip of the Reaction Vial with the tongs, and mix the solution vigorously for at least 30 seconds with the vortex.
8. Replace the Reaction Vial back into the lead shield.

E. Product Sterilization using Membrane Filtration (see Figure)

1. Place a 10-mL sterile empty vial into a lead shield. This is the Product Vial.
2. Swab the septum of the final product container with a sterile IPA pad.
3. Attach a 21-gauge needle to a Millex-GV Sterilizing Filter Unit.
4. Insert the needle of the filter-needle assembly through the septum of the Product Vial.
5. Attach a second 21-gauge needle to a second Millex-GV Sterilizing Filter Unit.
6. Insert the needle of the second filter-needle assembly through the septum of the Product Vial.
7. Attach the 33" extension tubing to one of the Filter Units on the Product Vial.
8. Attach the remaining end of the 33" extension tubing to a 10-mL syringe.
9. Attach the 6" extension tubing to the second Filter Unit on the Product Vial.
10. Using tongs and a sterile IPA pad, swab the septum of the shielded Reaction Vial.
11. Attach the second end of the 6" extension tubing to a 3 ½ "19-gauge spinal needle.
12. Insert the spinal needle through the septum of the Reaction Vial so that the needle touches the bottom of the vial.
13. Attach a 21-gauge needle to a Millex-GV Sterilizing Filter Unit.
14. Insert the needle of the filter needle assembly through the septum of the Reaction Vial.
15. Carefully draw back the plunger of the syringe to pull the solution from the Reaction Vial through the sterilizing filter and into the Product Vial (see diagram).
16. When the fluid transfer is complete, withdraw the needle-filter assemblies from the Product Container.
17. Remove the Millex-GV Sterilizing Filter Unit from the 6" extension tubing and perform membrane integrity testing.
18. Dispose of all radioactive waste and sharps in an appropriate manner.
19. Proceed to quality control testing of the contents of the Product Vial.
Note: Vials must be in lead shields (not shown)
10.2 Appendix 2

Preparation of $^{64}\text{Cu}$ -ATSM using Proportional Technologies, Inc. (PTI)
Kit Formulation (Version 1)

(Alternate Method)
10.2 Appendix 2


Index:
I. Overview
II. Training
III. Equipment
IV. Procedure

I. Overview
This document describes the procedure for the Version 1 preparation of $^{64}$Cu-ATSM using the Proportional Technologies, Inc. (PT) kit.

II. Training
This procedure can only be carried out by trained personnel under the supervision of the Authorized User(AU)/Principal Investigator(PI)/Authorized Nuclear Pharmacist (ANP) or his/her designees. A list of trained personnel will be kept by the AU/PI/ANP and available upon request. Personnel must understand and practice all relevant safety and security procedures.

III. Equipment
a. Equipment
   - Dose Calibrator
   - Vortex
   - Lead Shield for 50-mL Vial
   - Lead Shield for 10-mL Vial
   - Lead Shield for 10-mL Syringe
   - Tongs

b. Supplies
   - Proportional Technologies, Inc. (PTI) kit (consisting of Reconstitution Solution in 10-mL vial and Lyophilized ATSM Ligand in 50-mL Vial)
   - $^{64}$CuCl$_2$ solution in 0.1 M HCl [Supplied by Washington University in St. Louis in a 0.6 mL vial]
   - Two 0.22-μm Millex-GV Sterilizing Filter Units (Millipore) (or equivalent)
   - One 1-mL Luer-Lok syringe (Tyco Monoject® or equivalent)
   - Two 10-mL Luer-Lok syringes (Terumo® SS-10L or equivalent)
   - Five 21-gauge needles (Kendal Monoject® 1188 or equivalent)
   - One 10-mL Sterile Empty Vial (Hospira ABB5816-11 or equivalent)
   - Six Sterile 70% Isopropyl Alcohol (IPA) Pads (Kendall 6818 or equivalent)
IV. Procedure

A. Personal Protection
1. All personnel must don lab coats, eye protection, and disposable protective shoe covers before entering the laboratory.
2. Sleeve protectors and two pair of disposable gloves must be worn during the preparation.
3. All personnel must wear TLD radiation badges and rings.

B. Drug Compounding
1. It is recommended that radiopharmaceutical compounding proceed in a Class 100 environment.
2. Drug preparation should involve aseptic processing.
3. Principles of ALARA must be observed for all aspects of drug compounding.

C. Reconstitution of ATSM ligand
1. Swab the septa of the two PT kit vials with sterile IPA pads.
2. Attach a 21-gauge needle to a 10-mL syringe.
3. Withdraw 10 mL of the reconstitution solution into the 10-mL syringe.
4. Discard the empty syringe-needle assembly appropriately.
5. Inject the contents of the 10-mL syringe into the 50-mL vial containing the lyophilized ATSM ligand.
6. Mix the solution vigorously for at least one minute with the vortex.
7. Place the 50-mL vial into a lead shield. This is the Reaction Vial.

D. Radiolabeling of ATSM Ligand
1. Using a sterile IPA pad, swab the septum of the 50-mL vial containing the reconstituted ATSM ligand.
2. Using tongs and a sterile IPA pad, swab the septum of the $^{64}$CuCl$_2$ vial in the lead shield.
3. Attach a 21-gauge needle to a 1-mL syringe.
4. Withdraw the $^{64}$CuCl$_2$ solution into the 1-mL syringe.
5. Inject the contents of the 1-mL syringe into the shielded 50-mL Reaction Vial containing the reconstituted ATSM ligand.
6. Discard the empty syringe-needle assembly appropriately.
7. While holding the Reaction Vial with tongs, mix the solution vigorously for at least 30 seconds with the vortex.
8. Place the Reaction Vial back into the lead shield.

E. Product Sterilization using Membrane Filtration
1. Place a 10-mL sterile empty vial into a lead shield. This is the Product Vial.
2. Swab the septum of the Product Vial with a sterile IPA pad.
3. Attach a 21-gauge needle to a Millex-GV Sterilizing Filter Unit.
4. Insert the needle of the filter-needle assembly through the septum of the Product Vial. This is the vent assembly.
5. Using tongs, swab the septum of the Reaction Vessel with a sterile IPA pad.
6. Attach a 21-gauge needle to a 10-mL syringe.
7. Place the needle-syringe assembly into a lead syringe shield.
8. Withdraw the contents of the Reaction Vessel into the 10-mL syringe.
9. Attach a 21-gauge needle to a Millex-GV Sterilizing Filter Unit.
10. Replace the needle on the 10-mL syringe with the needle-filter assembly.
11. Insert the needle of the needle-filter-syringe assembly through the septum of the Product Vial.
12. Carefully inject the contents of the 10-mL vial through the filter into the Product Vial.
13. Remove the empty syringe-filter-needle assembly from the Product Vial.
14. Remove the Millex-GV Sterilizing Filter Unit from the empty syringe and perform membrane integrity testing.
15. Dispose of all radioactive waste and sharps in an appropriate manner.
16. Proceed to quality control testing of the contents of the Product Vial.
10.3 Appendix 3

Determination of Radiochemical Purity of $^{64}\text{Cu}$-ATSM using Paper Radiochromatography

(Preferred Method)
10.3 Appendix 3

Determination of Radiochemical Purity of $^{64}$Cu-ATSM using Paper Radiochromatography

Index:
I. Overview
II. Training
III. Equipment
IV. Procedure

I. Overview

This document describes the procedure for determining the radiochemical purity of $^{64}$Cu-ATSM by paper radiochromatographic analysis. A strip of chromatographic paper is spotted 2 cm from the bottom with a sample of the $^{64}$Cu-ATSM solution. The chromatographic strip is placed into a developing chamber containing a mobile phase of 100% ethyl acetate, and permitted to remain until the solvent front reaches 15 cm from the bottom of the strip. The chromatographic strip is then removed and analyzed to determine the percentage of total radioactivity that is $^{64}$Cu-ATSM (with Rf 0.9). The radiochemical purity of $^{64}$Cu-ATSM determined in this fashion should be no less than 95%.

II. Training

This procedure can only be carried out by trained personnel under the supervision of the Authorized User (AU)/Principal Investigator (PI)/Authorized Nuclear Pharmacist (ANP) or his/her designees. A list of trained personnel will be kept by the AU/PI/ANP and available upon request. Personnel must understand and practice all relevant safety and security procedures.

III. Equipment and Supplies

A. Equipment

TLC Developing Chamber, 29.3 cm x 27.5 cm x 9.3 cm (outside) (L x H x W)
(ScientificManufacturing Industries or equivalent)
Scissors
Metric Ruler
Pencil
Tongs
Radioactivity Counter or Radiochromatogram Scanner

B. Supplies

Chromatography Paper Sheet, 46 cm x 57 cm (Whatman 17 CHR, catalog no. 3017-915 or equivalent)
Sterile 70% Isopropyl Alcohol (IPA) Pad (Kendall 6818 or equivalent)
Micropipette, 0.5-10 µL (Eppendorf catalog no. 05-402-86 or equivalent)
Ethyl Acetate, HPLC Grade (EMD catalog no EX0245-1 or equivalent)
Snap-Cap Microcentrifuge Tube (Eppendorf Catalog no. 22600044 or equivalent)
Insulin Syringe (Terumo catalog no. SS*30M2913 or equivalent)

IV. Procedure

A. Personal Protection

1. All personnel must don lab coats, eye protection, and disposable protective shoe covers before entering the laboratory.
2. Sleeve protectors and two pair of disposable gloves must be worn during the preparation.
3. All personnel must wear TLD radiation badges and rings.

B. Preparation of Chromatographic Strip

1. With scissors, cut a 2.54 cm x 16 cm strip from the sheet of chromatography paper.
2. Using a metric ruler and pencil, make a very light mark at the bottom of the chromatographic strip at 1.0 cm from the edge for use as a guide when applying the sample.
3. Using a metric ruler and pencil, make very light marks on both sides of the strip at 2 cm from the bottom of the strip (see below). This will be the origin for the paper radiochromatographic analysis.
4. Using a metric ruler and pencil, make very light marks on both sides of the strip at 15 cm from the bottom of the strip (see below). This will be the solvent front for the paper radiochromatographic analysis.
Figure: Paper strip used for radiochromatographic analysis of $^{64}\text{Cu}}$-ATSM. Sample is placed at the origin, located 2 cm from the bottom of the strip. Solvent front is located 15 cm from the bottom of the strip. Width of the paper strip is 2.54 cm; length is 16 cm.

C. Preparation of the TLC Chamber

1. Pour 50 mL of ethyl acetate into the clean TLC chamber. The depth of the liquid will be approximately 5 mm.
2. Place the lid onto the chamber and permit to equilibrate for at least 30 minutes prior to use.

D. Preparation of $^{64}\text{Cu}}$-ATSM sample

1. Using tongs and a sterile IPA pad, swab the septum of the Product Vial.
2. With use of the insulin syringe, aseptically withdraw 100 µL of the contents of the Product Vial.
3. Empty the contents of the insulin syringe into the microcentrifuge tube.
E. Application of $^{64}$Cu-ATSM Sample

1. Remove 1.5 µL of the $^{64}$Cu-ATSM sample solution from the microcentrifuge tube.
2. Apply the 1.5 µL sample to the origin location (2 cm) of the chromatographic strip.
3. Allow the sample to dry 1-2 minutes under ambient conditions.

F. Chromatographic Development of $^{64}$Cu-ATSM Sample

1. With use of tongs, place the chromatographic strip bearing the 1.5 µL sample of $^{64}$Cu-ATSM into the TLC chamber containing ethyl acetate. Verify that the solution contacts the entire bottom of the chromatographic strip.
2. Note the rise of the mobile phase up the chromatographic strip.
3. Permit the ethyl acetate to rise to the 15 cm mark on the chromatographic strip. This takes approximately 15 minutes.
4. When the solvent reaches the top mark, remove the chromatographic plate from the chamber using tongs.
5. Dry the chromatographic strip. Verify that the strip is completely dry.

G. Determination of Radiochemical Purity of $^{64}$Cu-ATSM

1. Radiochromatographic Scanning
   a. Scanning the Chromatographic Strip
      i. Cover the dry chromatographic strip with plastic wrap.
      ii. Place the covered chromatographic strip onto the scanner bed.
      iii. Place the scanner head in the proper position to initiate data acquisition.
      iv. Initiate strip scanning.
      v. Save the scanned data into the scanner database, denoting the batch number, date and other pertinent information.
      vi. Upon conclusion of scanning, remove chromatographic strip from the scanner bed.
      vii. Place the chromatographic strip into a shielded area for decay.
   b. Analysis of the Radiochromatogram
      i. Using scanner software, retrieve the stored data for the appropriate batch.
      ii. Select regions of interest (ROI) corresponding to each radioactive peak on the radiochromatogram.
      iii. Select ROI corresponding to background on the radiochromatogram.
      iv. Integrate all peak ROI of the radiochromatogram.
      v. Calculate the corrected counts for each peak as
Corrected Peak ROI = Peak ROI – Background ROI
vi. Sum all Corrected Peak ROI
vii. Calculate the percentage of total activity in each peak as

% ROI = Corrected ROI / Sum ROI

viii. The % ROI for the peak corresponding to R_f 0.9 is the radiochemical purity for the batch of $^{64}$Cu-ATSM product. This value must be ≥ 95% to meet release specifications.

2. Radiochemical Purity (RCP) Measurement via Sectional Assay

a. Measurement of Radioactivity Distribution
   i. Using scissors, cut the dry chromatographic strip along its length into eight sequential 2-cm sections starting at 1 cm below the origin (2 cm mark) and ending at 1 cm past the solvent front (15 cm mark).
   ii. Place each 2-cm section into a test tube for radioactivity assay.
   iii. Assay the radioactivity content in each section.
   iv. Assay the background radioactivity.
   v. After completion of assay, place the radioactive strips into a shielded area.

b. Calculation of Radiochemical Purity (see Attached Worksheet)
   i. Correct the radioactivity in measured in each section for background:

      Corrected Section Activity = Section Activity – Background Activity.

   ii. Calculate the Corrected Peak Activity by adding together the corrected activity measurements for the sections 2 through 8.
   iii. Sum all corrected section activity measurements for the chromatographic strip.
   iv. Calculate the radiochemical purity (RCP) as:

      RCP = (Corrected Activity Section 2-8) / (Corrected Activity Section 1-8)

   v. The RCP must be ≥ 95% to meet release specifications.

Document results of radiopharmaceutical purity testing on the trial-specific record form.
## RADIOCHEMICAL PURITY TESTING OF ⁶⁴Cu-ATSM VIA PAPER CHROMATOGRAPHY

**Batch Number:** __________________

**Date:** __________________

**Name of Operator:** __________________

<table>
<thead>
<tr>
<th>Section Number (2-cm wide)</th>
<th>Measured Counts</th>
<th>Corrected Counts = (Measured Counts – Background Counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Unbound</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Bound</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Bound</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>Bound</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Bound</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Bound</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Bound</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Background</td>
</tr>
</tbody>
</table>

**Corrected Counts (Sections 2 through 8)**

**Corrected Counts (Sections 1 through 8)**

---

**RADIOCHEMICAL PURITY**

\[
= \frac{\text{Corrected Counts Sections 2-8}}{\text{Corrected Counts Section 1-8}} \times 100\%
\]

\[
= \frac{\left(\text{_________}\right)}{\left(\text{_________}\right)} \times 100\%
\]

\[
= \text{_________}%
\]
10.4 Appendix 4

Determination of Radiochemical Purity of $^{64}$Cu-ATSM using Oasis Cartridge

(Alternate Method)
10.4 Appendix 4


Index:

I. Overview
II. Training
III. Equipment
IV. Procedure

I. Overview
This document describes the procedure using Oasis cartridge for radiochemical purity determination of $^{64}$Cu-ATSM produced using the Proportional Technologies (PT) kit.

II. Training
This procedure can only be carried out by trained personnel under the supervision of the Authorized User/Principal Investigator (PI)/Authorized Nuclear Pharmacist (ANP) or his/her designees. A list of trained personnel will be kept by the AU/PI/ANP and available upon request. The preparer must understand and practice all relevant safety and security procedures.

III. Equipment and Supplies

A. Equipment
   Dose Calibrator

B. Supplies
   $^{64}$Cu-ATSM (PT) solution, as prepared in Appendix 1
   Three 5-mL Luer-Lok syringes, BD 5-mL syringe ref 309603 or equivalent
   Oasis Cartridge, Waters part no. 18600383 or equivalent
   Luer/Luer transfer set made by Proportional Technologies

Can be assembled from:
   Tubing: Cole-Parmer, Pharmed BPT tubing, 0.035" id x 0.099" od x 0.032" wall, part number 95809-26
   Clamp: Bel-art Products (belart.com), clamp, acetal, 100/pkg, catalog number 182270000
   There are two plastic fittings on the apparatus obtained from Value Plastics (valueplastics.com).
      FTL210-6, female Luer lug style to 200 series barb 1/16" id tubing, natural polypropylene
      MTLLP-6, male Luer integral lock ring plug, closed at Luer tip, natural polypropylene
IV. Procedure

A. Personal Protection
   1. All personnel must don lab coats, eye protection, and disposable protective shoe covers before entering the laboratory.
   2. Sleeve protectors and two pair of disposable gloves must be worn during the preparation.
   3. All personnel must wear TLD (or film) radiation badges and rings.

B. Oasis column preparation procedure:
   1. Install Luer/Luer transfer set to bottom of Oasis cartridge.
   2. Install a 5-mL syringe to transfer set. The figure below illustrates the proper Oasis cartridge set up.

   ![Oasis cartridge setup](image)

   3. Fill Oasis barrel with 1 mL ethanol and draw through over 10 seconds. Do not draw the entire volume through the Oasis filter; leave a meniscus on top of the filter.
   4. Fill Oasis barrel with 1 mL saline and draw through in similar manner. Draw the entire volume through the Oasis filter; leave a meniscus on top of the filter.
   5. Repeat step 4 and then disconnect the 5-ml receiving syringe.

C. Radiochemical purity assay can be performed by a complete radiochemical purity assay:
   1. Dispense <10 μL of the labeled sample into barrel of prepped Oasis cartridge. Add 500 μL of saline. (The new reconstitution solution contains an organic solvent. Do not add more than 10 μL of labeled solution onto the Oasis cartridge.)
   2. Draw labeled sample through resin bed into 5 mL syringe using continuous flow over 10 seconds load time. Draw the entire volume through the Oasis filter; leave a meniscus on top of the filter.
   3. Dispense 1 mL saline into barrel and draw into the same syringe at similar rate.
   4. Repeat step 3.
   5. Remove 5-mL syringe, cap and place in a plastic bag (labeled C2).
   6. Install a new 5-mL syringe to transfer set.
   7. Add 1 mL of ethanol into barrel and draw into the 5-mL syringe over 10
seconds load time. **Draw the entire volume through the Oasis filter, leave a meniscus on top of the filter.**

8. Repeat step 7 two more times.
9. Remove 5-mL syringe, cap and place in a plastic bag (labeled C3).
10. Place the Oasis cartridge in a separate plastic bag (labeled C1).
11. Assay the three bagged items for relative $^{64}$Cu activity in a dose calibrator.
12. Measure background (BKG).
13. Subtract BKG count from each of the three sample counts
14. Compute the radiochemical purity as follows.

   \[
   \text{C1} = \text{cartridge count} \\
   \text{C2} = \text{aqueous saline syringe count} \\
   \text{C3} = \text{ethanol syringe count}.
   \]

   \[
   \text{Radiochemical purity} = \left[ \frac{\text{C3}}{\text{C1} + \text{C2} + \text{C3}} \right] \times 100\%
   \]

15. The minimum acceptable radiochemical purity of the $^{64}$Cu-ATSM is 95%. The product should not be released for clinical use if the radiochemical purity is less than 95%.

**Document radiopharmaceutical purity testing on trial specific record form.**
10.5 Appendix 5

Batch Record Form: Process and Production Record
**Part 1. Materials and Equipment**

Note the lot numbers of the items used and obtain a verification check.

**Table A. Materials and Equipment**

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer</th>
<th>Lot Number</th>
<th>Expiration Date</th>
<th>Verified / Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{64}$CuCl$_2$</td>
<td>MIR, Cyclotron Facility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATSM lyophilized vial</td>
<td>PTI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reconstitution solution</td>
<td>PTI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.22 µm Sterile Filter Units</td>
<td>PTI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Part 2. Drug Preparation**

2. Method of $^{64}$Cu-ATSM Preparation

- [ ] Version 2 PTI Kit Formulation **Preferred Method** Complete Table B, then skip to Part 3
- [ ] Version 1 PTI Kit Formulation Skip to Table C

**Table B. Version 2 Method** *Per Appendix 1 of the Investigator’s Brochure*

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Verified / Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Record the volume and reference the time ATSM added to $^{64}$CuCl$_2$. Activity added ______ mCi Time ___ : ___</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Volume added ______ mL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sterile filtration performed</td>
<td>Time Completed ___ : ___</td>
</tr>
<tr>
<td>3</td>
<td>Radioactivity assay in Product Vial: ______ mCi Time of measurement ___ : ___</td>
<td></td>
</tr>
</tbody>
</table>

---

**Table C. Version 1 Method** *Per Appendix 2 of the Investigator’s Brochure*

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Verified / Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Record the volume and reference the time $^{64}$CuCl$_2$ added to ATSM kit. Activity added ______ mCi Time ___ : ___</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Volume added ______ mL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sterile filtration performed</td>
<td>Time Completed ___ : ___</td>
</tr>
<tr>
<td>3</td>
<td>Radioactivity assay in Product Vial: ______ mCi Time of measurement ___ : ___</td>
<td></td>
</tr>
</tbody>
</table>
Part 3. Radiochemical Purity

3. Method of $^{64}$Cu-ATSM Radiochemical Purity Measurement
   - Paper Chromatography Method  **Preferred Method** Complete Table D, then skip to Part 4
   - Oasis Cartridge Method Skip to Table E

Table D. Paper Chromatography Method  **Per Appendix 3 of the Investigator’s Brochure**

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Verified / Initials</th>
</tr>
</thead>
</table>
| 1    | Time of spotting: ________________  
      Start time of chromatographic development: ____ ____ : ____ ____  
      Finish time of chromatographic development: ____ ____ : ____ ____  
      Solvent front distance: ________________ |                      |
| 2    | Time of radioactivity analysis: ________________  
      Check method of analysis:         ☐ Radiochromatographic scanner  
                                         ☐ Sectional assay  
      *Retain radiochromatogram scans or section assay worksheet as source document.  
      Record results in Question 8.* |                      |

Table E. Oasis Cartridge Method  **Per Appendix 4 of the Investigator’s Brochure**  

**Skip to Part 4**

Table F. Oasis Cartridge Method

**Radiochemical Purity Measurement**

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Verified / Initials</th>
</tr>
</thead>
</table>
| 1    | Background dose calibrator reading: Activity ________________ µCi  
      Time ____ ____ : ____ ____ |                      |
| 2    | Dose calibrator measurements of fractions  
      a. Activity C1 __ __ __ : __ __ µCi  
      Net Activity __ __ __ : __  
      Time ____ ____ : ____ ____  
      b. Activity C1 __ __ __ : __ __ µCi  
      Net Activity __ __ __ : __  
      Time ____ ____ : ____ ____  
      c. Activity C1 __ __ __ : __ __ µCi  
      Net Activity __ __ __ : __  
      Time ____ ____ : ____ ____ |                      |
| 3    | Percentage purity of $^{64}$Cu-ATSM = C3/ (C1+C2+C3) x 100%  
      Radiochemical purity __ __ __ : __ %  
      *Also record results in Question 8.* |                      |

Part 4. Release Specifications for $^{64}$Cu-ATSM

Table F. Oasis Cartridge Method

<table>
<thead>
<tr>
<th>Test</th>
<th>Acceptance Criteria</th>
<th>Procedure</th>
<th>Testing result</th>
<th>Verified / Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiochemical Purity</td>
<td>≥ 95%</td>
<td>☐ Chromogenic method or ☐ Gel clot method</td>
<td>☐ Pass or ☐ Fail</td>
<td></td>
</tr>
<tr>
<td>Bacterial Endotoxin</td>
<td>≤ 175 EU/V (where V is the maximum total dose)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{64}$Cu-ATSM prepared by: ________________________________  
Date prepared: _____-____-____

QC performed by: ________________________________  
Date performed: _____-____-____

Data reviewed by: ________________________________  
Date reviewed: _____-____-____

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